

University of Alberta

Evolution and Assembly of a Plasmid DNA Conjugation Apparatus

by

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of the requirements for the degree of Doctor of Philosophy in Bacteriology

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ABSTRACT

Intercellular transfer of plasmid DNA molecules is achieved by a large array of proteins which assemble into both cytoplasmic and membrane-associated complexes. The incompatibility group H plasmids R478, R27, and pHCM1 are large conjugative genetic elements (≥ 180 kbp), are harboured within pathogenic bacteria, and encode resistance to multiple antibiotics and heavy metals. The evolution and function of the H plasmid transfer apparatus was investigated by comparative sequence analyses, fluorescent labeling of transfer protein complexes, and biochemical characterization of protein interactions.

The 274,762 bp sequence of R478 was determined and used to define the minimal set of core H plasmid determinants (including replication, partitioning, and conjugative transfer) by a comparative analysis with R27 and pHCM1 sequences. No resistance determinants are predicted to be present in the ancestor common to these plasmids, and a comparison with related transfer sequences suggested that the H-type transfer determinants encoding mating pair formation (Mpf) functions are of a different lineage than those determinants encoding DNA-processing (Dtr) and membrane-targeting (coupling) functions.

An assembly of R27-encoded proteins was visualized through an Mpf protein-green fluorescent protein fusion, which appeared as discrete membrane-associated fluorescent foci. Thirteen of the nineteen R27 transfer proteins were required for focus formation, and an individual focus possibly represents a subassembly comprised of some or all of these transfer proteins. These data support the notion that the transfer apparatus is a multi-component structure. The fluorescence assay also demonstrated that the NTP-binding motifs of the putative ATPase TrhC were necessary for conjugative transfer, but not for assembly of the fluorescent subcomplex, and that the temperature-sensitive

phenotype of H plasmid transfer results from temperature-dependent expression of R27 transfer genes.

The proposed link between the Mpf and Dtr protein complexes is the coupling protein. This model was confirmed by bacterial two-hybrid and immunoprecipitation assays which characterized a direct interaction between the R27-encoded coupling protein TraG and the Mpf protein TrhB. Evolution of the H-type transfer system required the adaptation of unrelated Mpf and Dtr determinants by the coupling protein to form an apparatus functional for conjugative plasmid transfer.

'Dealing with ancient events of necessity involves conjecture' – R.F. Doolittle

For my parents, Warren and Janice Gilmour,
and grandparents, George and Mildred Boyce

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LIST OF ABBREVIATIONS

Ap	ampicillin
BLAST	basic local alignment search tool
bp	base pairs
BTH	bacterial two-hybrid
CDS	coding sequence
Cm	chloramphenicol
DNA	deoxyribonucleic acid
dr	derepressed
Dtr	DNA transfer replication
DTT	dithiothreitol
FPLC	fast protein liquid chromatography
GFP	green fluorescent protein
HRP	horseradish peroxidase
IPTG	isopropyl- β -D-thiogalactoside
Inc	incompatibility group
Km	kanamycin
LB	Luria-Bertani
MDR	multiple drug resistant
Mpf	Mating pair formation
Nal	nalidixic acid
NTP	nucleotide triphosphate
NCBI	National Center for Biotechnology Information
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PSI-BLAST	position specific iterative – basic local alignment search tool
Rif	rifampicin
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
T3S	Type III secretion
T4S	Type IV secretion
Tc	tetracycline

TMH	transmembrane helix
Tra1	transfer region 1
Tra2	transfer region 2
Tris	Tris (hydroxymethyl) aminomethane
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Chapter 1

General Introduction

1. General Introduction

1.1 The tree of life

The three domains of life include the Eucarya, the Bacteria, and the Archaea, with members of the latter two domains collectively called prokaryotes (Woese *et al.*, 1990). A visual representation of this classification scheme presents each domain as being derived from a universal common ancestor, and an absence of genetic exchange since the divergence of these lineages. This phylogenetic structure is called into question by the growing evidence from genome sequencing projects that transfer of genetic material occurs between members of the same domain, and between members of different domains (Doolittle *et al.*, 2003). General examples include the observation of Archaeal coding sequences in Bacterial genomes, or vice versa. It is possible that orthologous genes present in multiple domains may have been inherited from the universal common ancestor, however if true for all such circumstances, this theoretical ancestral genome would exceed a reasonable size (Koonin, 2003). Instead, it is more plausible that some genes have independently evolved and subsequently transferred between genomes. Such events represent lateral or horizontal gene transfer, and these blur the lines that comprise the universal tree of life.

Gene transfer events have predominantly been reported between prokaryotic genomes (Doolittle *et al.*, 2003). This bias is likely a result of the uni-cellular nature and reproductive strategy of prokaryotes (Lawrence, 2002). Eukaryotes propagate in most instances through sexual reproduction, wherein two distinct meiotic genomes are combined to create a progeny unique from (but representative of) the parental genomes. Conversely, prokaryotic cells generate progeny through binary fission after faithful replication of their own chromosomal and extra-chromosomal genetic elements, without the requirement of genetic input from a second parent cell. In addition, cell fusion, as required during eukaryotic reproduction is prohibited by the peptidoglycan exoskeleton

possessed by bacterial cells (Cavalier-Smith, 2002). In the absence of gene transfer, the only diversity introduced into a prokaryotic genome would be errors (i.e. point mutations) or small re-arrangements introduced during DNA replication. The evolutionary force of point mutations to create new species would eventually be seen over the course of millennia, but the immediate effect would be genomic stasis. Alternatively, the integration of 'foreign' genetic determinants (possibly encoding novel functions) into the genome of a prokaryote would result in the inheritance of those determinants after a single generation. For a multi-cellular organism, germ line cells would be the only recipients which would perpetuate transferred genetic information. Furthermore, the only confirmed eukaryotic recipients of trans-domain transfer events have been protists (Doolittle *et al.*, 2003).

The degree of horizontal gene transfer amongst prokaryotes lies somewhere between two extremes: the complete absence of transfer, or a singular 'bacterial superbiosystem' (Sonea and Mathieu, 2001) in which all genes might be freely accessible and transferable. Numerous limitations to gene transfer prevent the occurrence of the latter possibility, including geographical barriers, the inefficiency of recombination between unrelated sequences, the bacterial mis-match repair system, and incompatibility of genetic features such as promoter and termination sequences and codon usage biases between different organisms (Lawrence and Hendrickson, 2003; Majewski *et al.*, 2000). The most obvious physical barrier which must be overcome during horizontal transfer is the bacterial envelope, and there are multiple strategies to transfer DNA molecules into bacterial cells.

1.2 Mechanisms of horizontal gene transfer

The three known mechanisms of horizontal genetic exchange are transduction (by bacteriophage), transformation (by naked DNA), and conjugation (by plasmids) – each

capable of transmitting DNA across the bacterial cell envelope. A now-classic example of the evolutionary consequences of horizontal gene transfer is the conversion of the benign bacterium *Escherichia coli* to a pathogenic strain through the acquisition of pathogenicity determinants (Perna *et al.*, 2001). It was estimated that 26% (1.34 Mbp) of the genome of the reference enterohemorrhagic O157:H7 strain *E. coli* EDL933 is not present in the lab strain K-12, and may represent horizontally transferred sequences (Perna *et al.*, 2001). This includes 338 genes that are predicted to be of bacteriophage origin, and in particular, the Shiga-like toxin (Stx2) sequence represents such a prophage determinant (Datz *et al.*, 1996; Perna *et al.*, 2001). Other pathogenicity determinants, such as the locus for enterocyte effacement present in numerous pathogenic *E. coli* strains, and additional adhesion, toxin and secretion systems have also been acquired by mechanisms of horizontal gene transfer (Perna *et al.*, 2001).

The evolution of pandemic *Vibrio cholerae* strains also resulted after transduction of pathogenicity determinants (Faruque and Mekalanos, 2003). *V. cholerae* strain O139 encodes numerous phage pathogenicity islands, including the CXT ϕ element which carries the cholera toxin genes (Waldor and Mekalanos, 1996). As bacteriophage cycle through bacterial hosts, it is possible that DNA from the current host bacterium is packaged and transmitted to subsequent hosts, as well as the phage DNA (Birge, 1994). Such gene transfer events represent transduction, and the range of gene transfer is limited by the amount of DNA packaged by the phage and the different bacteria which can be infected (Ochman *et al.*, 2000). During transduction, bacteriophage may recognize specific features on, and gain entry into the cell via the extracellular pili or the associated pilus assembly apparatus (Grahn *et al.*, 1997; Paranchych, 1975). The genomic sequence data from Proteobacteria (including *E. coli* and *V. cholerae*) reveal that in most instances multiple (7-20) prophages are present (Casjens, 2003).

The first example of genetic exchange, and one of the seminal experiments to demonstrate that DNA stores genetic information, was the natural transformation and associated competence of *Streptococcus pneumonia* (Griffith, 1928). Natural transformation is the process by which bacterial cells are able to take up and maintain extracellular DNA without aid of chemical treatments (i.e. CaCl_2 -induced competence) (Dubnau, 1999). Because DNA is sampled from the environment, there is the possibility of acquiring distantly related genetic determinants (Ochman *et al.*, 2000). Limitations to this process include the recognition of specific uptake sequences (although this is not observed in all competence systems) and the capacity to incorporate heterologous sequences (Dubnau, 1999). The transformation proteins are similar to the pilus assembly proteins or bacterial conjugation proteins, each of which form multi-component transenvelope structures (Dubnau, 1999; Lawley *et al.*, 2003b). In particular, the competence system of the pathogens *Helicobacter pylori* and *Campylobacter jejuni* are related to proteins of the conjugative transfer apparatus (Bacon *et al.*, 2002; Hofreuter *et al.*, 2001).

The evolutionary significance of horizontal transfer was first highlighted by observations that antibiotic resistance determinants were spread through the bacterial gene pool by plasmid DNA molecules, rather than *de novo* synthesis of these features in each resistant isolate (Davies, 1994; Watanabe, 1963). These extrachromosomal genetic elements, capable of intercellular transfer, may confer upon the host cell (and recipients of a transfer event) several resistance phenotypes, including resistance to antibiotics, toxic heavy metals, colicins and bacteriophage. Notably, most pre-antibiotic era bacteria were not found to contain transferable resistance plasmids (Hughes and Datta, 1983), therefore the spread of resistance determinants to pathogens by conjugative plasmids seems to have occurred in response to human activity.

Among the essential features of conjugative plasmids are gene products and cis-acting elements for plasmid replication, partitioning and conjugal transfer, which are collectively termed the backbone or core functions (Thomas, 2000). Through these components, bacterial plasmids are both stable and transmissible within bacterial populations, and contribute to bacterial genetic exchange. Conjugative transfer is achieved by a macromolecular assembly of proteins which span the bacterial envelope and terminate in the extracellular space with a filamentous pilus.

1.3 Type IV secretion systems

Bacterial conjugation, in addition to being a mechanism of genome evolution, can be considered as a mechanism of macromolecular secretion. In particular, plasmid conjugative transfer is classified as a type IV secretion (T4S) system and represents the only known bacterial system for secretion of DNA (Christie, 2001). Bacteria not only transfer genetic information, such as determinants encoding antibiotic and heavy metal resistance using T4S systems; but also transfer virulence determinants into eukaryotic cells using these machines. Despite being encoded by a diverse range of bacterial genomes, each transferring a different protein or nucleoprotein substrate, these multi-component modules are each classified as T4S systems because there is conservation of both transfer gene sequences and organization (Christie, 2001; Nagai and Roy, 2003). In particular, components of the membrane-associated conjugative apparatus required for plasmid DNA transfer are similar to proteins responsible for the intercellular translocation of virulence factors from pathogenic Gram negative bacteria (Baron *et al.*, 2002).

Those T4S systems involved in pathogenesis have been subcategorized as 'effector translocators' by Cascales and Christie (2003). An increasing number of such effector translocation systems have been reported recently, including the prototypical secretion

of CagA by *H. pylori* which results in a hummingbird phenotype (through cytoskeletal rearrangements) in epithelial cells (Segal *et al.*, 1999), pertussis toxin secretion by *Bordetella pertussis* resulting in pleiotropic effects after delivery to mammalian cells (Farizo *et al.*, 2000), and plant oncogenesis mediated by the T-DNA nucleoprotein complex comprising proteins VirD2 and E2 secreted by *Agrobacterium tumefaciens* (Vergunst *et al.*, 2000). Other systems include the secretion of RalF by *Legionella pneumophila*, and unknown effectors released by *Bartonella* and *Brucella* spp., each necessary to maintain the lifestyle of these intracellular pathogens (Nagai *et al.*, 2002; O'Callaghan *et al.*, 1999; Schmiederer *et al.*, 2001; Seubert *et al.*, 2003b).

The T4S effector translocation systems have been compared to the type 3 secretion (T3S) systems, also involved in delivering protein effectors during pathogenesis (Cascales and Christie, 2003). Bacteria encoding T3S systems have been visualized with a needle-like extracellular superstructure that is anchored in the cell envelope by a structure resembling the flagellar basal body (Blocker *et al.*, 2001; Kubori *et al.*, 1998). Until recently, the only visible structure associated with T4S was the pilus. However, microscopic studies of *H. pylori* have revealed a filamentous structure having a central channel and covered in a sheath comprised of proteins related to *Agrobacterium* T4S proteins (Rohde *et al.*, 2003; Tanaka *et al.*, 2003). The apparent polar localization of this appendage on *H. pylori* cells also corresponds with the polar localization of the *Agrobacterium* pilus and T4S proteins (Kumar and Das, 2002; Lai *et al.*, 2000; Rohde *et al.*, 2003). The *H. pylori* filamentous appendage may be functionally analogous to the pilus, and have roles in cellular adhesion and/or substrate transfer.

T4S systems can also contribute to pathogenesis by releasing or transferring DNA which encodes virulence determinants. The human pathogen *Neisseria gonorrhoea* is naturally competent for DNA uptake, and DNA is released from donor gonococcus by a putative T4S system homologous to the F plasmid transfer system. These determinants

are encoded on a genomic island which also encodes a peptidoglycan hydrolase that likely contributes to inflammation by producing a peptidoglycan cytotoxin (Dillard and Seifert, 2001; Ding *et al.*, 2003; Hamilton *et al.*, 2001). This putative T4S system may be responsible for the transmission of this genomic island and other determinants amongst *Neisseria* spp. since the T4S and hydrolase determinants were each necessary for DNA secretion (Dillard and Seifert, 2001; Hamilton *et al.*, 2001). Conversion of avirulent *L. pneumophila* to a virulent strain was also reported to occur as a result of a T4S process (Miyamoto *et al.*, 2003). The *icm-dot* determinants (encoding T4S effector translocation systems) were transferred by a conjugative mechanism, and these data further highlight the evolutionary contributions made by horizontal gene transfer mechanisms.

1.4 Bacterial conjugation

Conjugative resistance plasmids are the paradigm for horizontal transfer between bacterial cells. In order to process and transport plasmid DNA, a multitude of cytosolic and membrane-associated proteins are required. Conjugal transfer proteins are believed to form at least three macromolecular complexes: the transenvelope mating pair formation (Mpf) complex, the cytosolic proteins responsible for initiating DNA transfer and replication (Dtr), and the coupling protein which is proposed to localize the Dtr components to the Mpf complex at the cell membrane through protein interactions with each complex (Schroder *et al.*, 2002; Willetts, 1981). In a widely accepted model for plasmid transfer the Mpf complex is responsible for production of the conjugative pili, which, after binding to an appropriate recipient cell, would retract via subunit depolymerization, thereby bringing both donor and recipient cells into close contact (Curtiss, 1969; Ou and Anderson, 1970). The exact nature of the conjugative pore through which DNA can traverse the cell wall of both donor and recipient bacteria is unknown.

1.4.1 Mating pair formation proteins

The Mpf complex represents a protein assembly analogous to the effector translocation T4S systems. This superstructure is hypothesized to contain both inner and outer membrane proteins, as well as periplasmic proteins, and could therefore span the cell envelope. Known functions of the Mpf complex include the production of conjugative pili and the entry of bacteriophage. The Mpf complex may also represent the route through which DNA traverses the cell envelope of the donor cell during conjugation. The associated pilus may be involved solely in attachment/recognition of recipient cells, or may additionally act in a needle-like fashion and inject the transferring DNA through the recipient cell wall.

The Mpf proteins encoded by plasmids F and RP4 are some of the best characterized of the known conjugative systems (Frost *et al.*, 1994; Lessl *et al.*, 1992a). Lanka and colleagues have identified 11 essential Mpf proteins by determining the role of each gene for RP4 transfer, mobilization of the non-self-transmissible plasmid RSF1010, pilus production, and phage propagation (Grahm *et al.*, 1997; Haase *et al.*, 1995; Lessl *et al.*, 1993). These same Mpf proteins, with the exception of TrbB, have been localized to a membrane fraction of intermediate density between inner and outer membrane fractions in cells containing RP4 or a Mpf-producing derivative, suggesting that the Mpf proteins assemble into a complex capable of joining these two bacterial membranes (Grahm *et al.*, 2000). Additionally, production of the core Mpf proteins in donor cells results in an electron-dense interface (termed conjugative junction) between cells, as observed during transmission electron and light microscopy (Durrenberger *et al.*, 1991; Samuels *et al.*, 2000). This likely represents the route through which DNA passes to the recipient cell, as there is cell-cell contact mediated by the Mpf complex. Furthermore, the point of fusion between mating pairs of donor and recipient bacteria is

located along the lateral wall or at the extreme ends of either cell (Lawley *et al.*, 2002b). These data suggest that sites for plasmid transfer are present throughout the cell periphery.

1.4.2 DNA transfer replication components

Processing of plasmid DNA prior to and during transfer resembles plasmid rolling circle replication (Lawley, 2003), as two double-stranded copies of a plasmid are ultimately produced after cleavage of the parental plasmid to generate two single-stranded templates. One of these single DNA strands is transferred to the recipient cell during bacterial conjugation, and the other strand remains in the donor cell. Processing of the plasmid to create the single-stranded transfer intermediate is the role of the relaxase – a member of the cytoplasmic relaxosome. This complex is also comprised of other DNA-binding proteins (such as integration host factor: IHF) which act at the origin of transfer (*oriT*), and cumulatively represent the Dtr aspects of bacterial conjugation (Nunez and De La Cruz, 2001). The *oriT* is generally an AT rich segment represented as a tandem of repeats containing a *nic* site where the relaxase cleaves a single strand of the DNA duplex. The nucleophilic tyrosine of the relaxase remains covalently bound to the 5' end of the cleaved strand (which will serve as the transferred strand), however an equilibrium between cleaved and uncleaved states exists, and the plasmid retains superhelicity (Guasch *et al.*, 2003; Pansegrau and Lanka, 1996). If a transfer event is initiated, the remaining free 3'-OH of the cleaved strand serves to initiate replacement strand synthesis using the host replication machinery and the uncleaved strand as a template. After transfer of the single DNA strand to the recipient is complete, both the replacement strand and transferred strand are recircularized by the relaxase (Zechner *et al.*, 2000). In the recipient cell, the transferred strand is converted into a double-stranded molecule after synthesis of nucleotide primers complementary to the

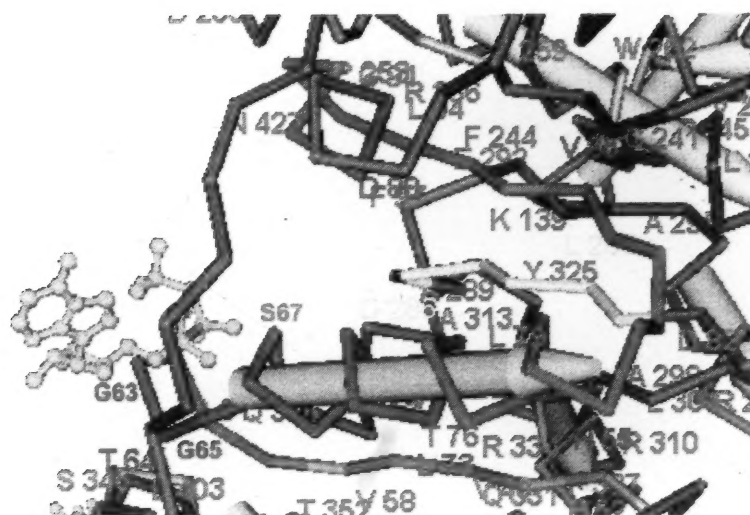
transferred strand, and containing a free 3'-OH from which complementary strand synthesis is initiated (Becker and Meyer, 2000; Pansegrau and Lanka, 1996).

1.4.3 Type IV coupling proteins

The DNA/relaxosome nucleoprotein complex is targeted to the inner membrane through a specific interaction with an integrally-associated membrane protein, termed the coupling protein (Schroder *et al.*, 2002; Szpirer *et al.*, 2000). This protein likely represents the inner membrane gate for transfer of substrate from the cytosol to the uncharacterized transenvelope structure, as suggested by the structure of the plasmid R388 coupling protein TrwB (Gomis-Ruth *et al.*, 2001). Using a TrwB truncation lacking the first 70 amino acids, a hexameric structure with a 20 Å central channel was revealed, including a nucleotide-binding domain in the central region and an all-alpha helical segment at the C-terminus. This quaternary structure resembles that of DNA ring helicases (such as FtsK), supporting the notion that DNA traverses through the central channel (Gomis-Ruth *et al.*, 2001). TrwB and other coupling proteins TraG (IncP plasmids), TraD (F plasmid), and HP0524 (*H. pylori*) have been shown to have non-specific DNA-binding activity (Schroder *et al.*, 2002). This may enable either docking of the DNA/relaxosome complex, and/or transfer of DNA through the central channel.

The individual proteins that comprise the different conjugative systems have conserved motifs, and in particular, Walker-type nucleotide triphosphate (NTP) binding domains [motif A, GxxGxGKS/T; motif B, hhhhDE; h=hydrophobic (Schneider and Hunke, 1998)] are the most common. The Walker A domain (or P loop) is involved in making contacts with the β - and γ -phosphates of ATP (Figure 1-1), whereas the Walker B domain binds divalent cations associated with the nucleotide (Schneider and Hunke, 1998). Each member of the coupling protein family have these domains, but none have been shown to hydrolyze ATP *in vitro* (Schroder *et al.*, 2002). ATP hydrolysis is

Figure 1-1. The structure of the Walker A and B domains associated with ATP. The three-dimensional structure of TrwB (structure accession: 1GL7_A) was visualized using the NCBI structure viewing and comparison program Cn3D after primary sequence alignment with R27-encoded TraG (red = identical, blue = similar, grey = unaligned). The Walker A domain (GATGTGKS) is numbered 60-67 and is present as the loop associating with ATP analogue (green) prior to the helical segment (purple). The yellow residues represent the Walker B domain (WLFIDE).



predicted to be an *in vivo* activity of the coupling proteins, resulting in structural rearrangements within this inner membrane gate and possibly energizing substrate translocation through the inner membrane to the Mpf complex (Gomis-Ruth *et al.*, 2002a; Gomis-Ruth *et al.*, 2002b).

All known plasmid conjugation systems encode a coupling protein, as well as the CagA (*H. pylori*) and T-DNA/Vir (*A. tumefaciens*) T4S effector translocation systems. The coupling protein may be involved in transporting effectors across the inner membrane, as demonstrated by an interaction between the *A. tumefaciens* VirE2 effector and VirD4 coupling protein (Atmakuri *et al.*, 2003). Other effector translocation T4S systems may circumvent the requirement of a coupling protein for inner membrane transport by using the general secretory system, as in *B. pertussis* Ptl secretion.

The function of the coupling protein may not be limited to the inner membrane targeting and transfer of substrates. In addition to making contacts with cytoplasmic relaxase and effector molecules, an interaction with the Mpf complex or T4S apparatus would deliver the transferring substrate to these envelope-spanning structures, thereby facilitating substrate export to recipient cells. As of yet, there is no evidence supporting a direct interaction between a coupling protein and Mpf/T4S protein.

1.5 Incompatibility group H plasmids

Plasmids of the *Enterobacteriaceae* are categorized into groups based upon their ability (or inability) to co-exist within the same host bacterial cell. These incompatibility groups are each comprised of plasmids which share common features necessary for replication and/or partitioning, and thus one plasmid species will ultimately be favored when multiple related DNA species are present in a single cell. Plasmids of the H incompatibility group (InCH) were first characterized after an outbreak of chloramphenicol-resistant typhoid fever in Mexico in 1972 (Anderson and Smith, 1972). H plasmids are

divided into two subgroups: HI and HII. Most of the IncHI plasmids are large genetic elements (>150 kbp), thermosensitive for transfer, frequently associated with human pathogens, encode serologically related pili, and carry antibiotic and heavy metal resistance determinants (Taylor, 1989). The IncHI group was further subdivided into IncHI1, IncHI2 and IncHI3 subgroups following restriction digestion, DNA-DNA hybridization studies, and incompatibility studies which each indicated that members of the HI group shared little DNA similarity (Roussel and Chabbert, 1978; Smith *et al.*, 1973; Whiteley and Taylor, 1983). In contrast to IncHI1 plasmids, IncHI2 plasmids, including R478, are compatible with the F plasmid and this depends on the lack of the RepFIB replicon which is found in both F and IncHI1 plasmids (Smith *et al.*, 1973; Taylor *et al.*, 1985).

IncH plasmids have historically been the sole source of multi-drug resistance (MDR) in *Salmonella enterica* serovar Typhi. The sequences of the IncHI1 resistance plasmids R27 (180 kbp) and pHCM1 (218 kbp) have already been determined (Parkhill *et al.*, 2001; Sherburne *et al.*, 2000). These two plasmids were isolated from different strains of *S. enterica* sv. Typhi over three decades apart and in different regions of the world (R27: England, 1961; pHCM1: Vietnam, 1993), yet over 168 kbp of each sequence are >99% identical (Parkhill *et al.*, 2001). The difference in size is attributed to two clusters of drug resistance determinants present in pHCM1, but not in R27, which may have been acquired and maintained due to selective pressure imposed by exposure to antibiotics. In a recent study, all examined MDR strains of *S. enterica* sv. Typhi contained a plasmid resembling pHCM1, and some demonstrated acquisition of additional resistance determinants (Wain *et al.*, 2003). Several other recent articles have documented the occurrence of Asian and Indian MDR *S. enterica* sv. Typhi isolates containing IncHI1 plasmids (Mirza *et al.*, 2000; Shanahan *et al.*, 1998; Shanahan *et al.*, 2000). This incompatibility group of plasmids has also been isolated from individuals

infected with *S. enterica* sv. Typhi living in Africa (Kariuki *et al.*, 2000) and travelers returning from South Asia to North America (Harnett *et al.*, 1998).

One of the characteristic features of IncHI plasmids is a thermosensitive mechanism of conjugation. Since transfer occurs only at lower temperatures (22°C to 30°C) and not at 37°C, it is believed that transfer is optimized to occur in soil and water environments (Maher and Taylor, 1993). IncHI plasmids are one of the only known plasmid groups harboured by human pathogens that are temperature sensitive for transfer.

The IncHI1 plasmid R27 transfer determinants arose from multiple origins, as there is a mosaic of IncF-like and IncP-like elements (Lawley *et al.*, 2002a; Sherburne *et al.*, 2000). The Mpf system of this resistance plasmid is a member of a growing subset of F-like type IV secretion systems, including those from R391, the *Vibrio cholera* SXT element, and the IncHI2 plasmid R478 (Beaber *et al.*, 2002; Boltner *et al.*, 2002; Lawley *et al.*, 2003a). Analogous to the IncP plasmids, the R27 genes encoding Dtr and Mpf functions are located in separate regions of the plasmid, termed Tra1 and Tra2 respectively. The Tra1 region encodes relaxosome components, an origin of transfer, and some Mpf proteins (Lawley *et al.*, 2002a); and the Tra2 region encoding the majority of the R27 Mpf proteins (Lawley *et al.*, 2003a; Rooker *et al.*, 1999). In total, 16 Mpf proteins, 3 relaxosome-associated proteins, and a coupling protein (TraG) are encoded by R27.

1.5.1 TrhC, a putative ATPase

Encoded within the R27 Tra2 region is *trhC*, a gene which is essential for R27 transfer as determined by transposon mutagenesis (Taylor *et al.*, 1999). TrhC was previously reported to contain 630 amino acids (Taylor *et al.*, 1999), but is now known to have 893 amino acids and is designated as a member of the VirB4 family of Type IV secretion proteins, which includes among others, VirB4 from the Ti plasmid, TrbE from

plasmid RP4, and TraC from the F plasmid. Of these, TrhC has the closest sequence homology to TraC: 22.5% identity over 888 contiguous amino acids. All of these proteins contain the A and B-type nucleotide-binding motifs, and a considerable degree of sequence identity at the C-terminus (Rabel *et al.*, 2003).

It has been speculated that the NTP-binding motifs of the VirB4 proteins act to power either the assembly of the transfer apparatus or translocation of substrate (Zechner *et al.*, 2000). Similar to the *in vivo* results of purified coupling proteins, preparations of the VirB4-related proteins TrbE and TrwK encoded by plasmids RP4 and R388, respectively, also did not hydrolyze ATP or GTP (Rabel *et al.*, 2003). ATP hydrolysis by TrbE and VirB4 may occur under *in vivo* conditions since the NTP-binding motifs are essential for the function of both proteins (Berger and Christie, 1993; Fullner *et al.*, 1994; Rabel *et al.*, 2003). The precise role of the NTP-binding motifs of VirB4-related proteins in the conjugative donor apparatus is ill-defined.

The subcellular location of the VirB4-like proteins has been examined. Using PhoA fusions, VirB4 was found in the cytoplasmic membrane with two transmembrane domains (Dang and Christie, 1997). The C terminal tail of VirB4 remained in the bacterial cytoplasm and the membrane-association of VirB4 was independent of other T-DNA transfer proteins. Likewise, RP4 TrbE associates with the cytoplasmic membrane in the absence of all other Mpf proteins, however when the entire Mpf complex is present, TrbE will also associate with the outer membrane (Grahm *et al.*, 2000). The TraC protein required for F plasmid transfer is a cytoplasmic protein when expressed in the absence of other Tra proteins, but when accompanied by the remainder of the transfer proteins, TraC then localizes to the cytoplasmic membrane (Schandel *et al.*, 1992). The subcellular localization of TrhC has not been experimentally determined, but it is an essential R27 Mpf protein with sequence identity to the VirB4 family of proteins,

and when in the context of the Mpf complex, therefore likely to be present in the bacterial membrane.

1.6 Objectives

Through bioinformatics, biochemistry, and cell biology it was the aim of this thesis to characterize the H plasmid conjugative transfer protein apparatus. From the sequence of the H plasmid transfer determinants it was apparent that the Mpf genes have a different origin than the Dtr and coupling determinants. The function of the conjugative apparatus, however, is dependent on the interaction between members of these different groups, as demonstrated by specific protein-protein interactions and the visualization of a transfer protein subcomplex. For the members of these different lineages to function together, it can be postulated that they were adapted to each other through a single protein – the coupling protein TraG, which also serves as the inner membrane pore. A model for the assembly of a transenvelope structure responsible for secretion of both DNA and proteins is also presented. This work will help define the mechanism and structures associated with DNA transfer.

Chapter 2

The complete nucleotide sequence of R478: defining the backbone components of incompatibility group H conjugative plasmids

Portions of this chapter have been submitted for publication as:

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* note: N.R. Thomson, M. Sanders, and J. Parkhill determined the complete nucleotide sequence of R478 and performed the initial sequence annotation at the Sanger Centre, Cambridge, UK.

2. The complete nucleotide sequence of R478: defining the backbone components of incompatibility group H conjugative plasmids

2.1 Introduction

The large resistance plasmid R478 was isolated in the USA in 1969 from a clinical isolate of *Serratia marcescens* (Medeiros and O'Brien, 1969); and was originally classified as an S plasmid in reference to this genus (Hedges *et al.*, 1975). In 1977, Taylor and Grant re-classified R478 into group HI2, and at this time, resistance to chloramphenicol, kanamycin, tetracycline, and mercuric ions was known to be mediated by this genetic element (Taylor and Grant, 1977a). IncHI2 plasmids were also known to inhibit the development of bacteriophages λ , T1, T5, and T7 (Taylor and Grant, 1977b). The thermosensitive profile of R478 conjugal transfer was determined to be optimal between 22-30°C, and inhibited at 37°C (Rodriguez-Lemoine *et al.*, 1975). Some R478-encoded determinants for replication, conjugative transfer and tellurite, arsenic and silver resistance have previously been described (Gupta *et al.*, 2001; Page *et al.*, 1999, 2001; Ryan and Colleran, 2002; Taylor, 1999; Whelan *et al.*, 1994; Whelan *et al.*, 1995).

In this study R478 was sequenced and annotated to identify the function and evolutionary history of IncHI plasmid backbone and accessory genes. Comparisons with the sequence data of R478, R27 and pHCM1, which have common backbone features but were isolated at different geographical and chronological points, allowed us to investigate the evolution of plasmid genomes.

2.2 Experimental Procedures

2.2.1 Sequencing and assembly of R478

The complete sequence of R478 was obtained from 6,644 paired end sequences (giving 11-fold coverage) derived from two pUC18 shotgun libraries (with insert sizes of 1.4 to 2 kb and 2 to 4 kb) using dye terminator chemistry on ABI3700 automated sequencers. All identified repeats were bridged by read-pairs or end-sequenced PCR

products. The sequence was assembled, finished and annotated as described previously (Parkhill *et al.*, 2000), using the program Artemis (<http://www.sanger.ac.uk/Software/Artemis>) to collate data and facilitate annotation (Rutherford *et al.*, 2000).

2.2.2 Annotation of R478 sequence data

Initial coding sequence predictions were performed using Orpheus (Frishman *et al.*, 1998) and Glimmer2 software (Delcher *et al.*, 1999). The two predictions were amalgamated, and codon usage (Nakamura *et al.*, 2000), positional base preference methods and comparisons to the non-redundant protein databases using BLAST (Altschul *et al.*, 1990) and FASTA (Pearson and Lipman, 1988) software were used to curate the predictions. Protein motifs were identified using Pfam (Bateman *et al.*, 2002), the CDD (Marchler-Bauer *et al.*, 2003), and Prosite (Falquet *et al.*, 2002), transmembrane domains were identified with TMHMM (Krogh *et al.*, 2001), and signal sequences were identified with SignalP version 2.0 (Nielsen *et al.*, 1997). The full-length sequence and annotation of plasmid R478 has been deposited in the EMBL database under accession number: BX664015. Comparison of the genome sequences was facilitated by using the Artemis Comparison Tool (ACT) (K. Rutherford, unpublished) which enabled the visualization of TBLASTX comparisons (Altschul *et al.*, 1990) between the genomes. Orthologous proteins were identified as reciprocal best matches using FASTA with subsequent manual curation (Table A-1). Additional local and global alignments were generated with ALIGN (at the EMBL website) using the BLOSUM62 matrix. Multiple sequence alignments were generated using ClustalW in MegAlign (DNASar) and GENEDOC for visual representation.

2.3 Results and Discussion

2.3.1 Physical features

The complete DNA sequence of plasmid R478 was obtained by a whole-plasmid shotgun method (Parkhill *et al.*, 2000), and it was determined to be a circular element of 274,762 bp. From the R478 sequence data, 295 coding sequences (numbered SMR0001-0304) representing a coding percentage of 83.5% were predicted (Figure 2-1). Homologous coding sequences were identified and are reported in Table A-1. The base composition of R478 is 45.5% G+C, whereas the relative buoyant density of R478 previously indicated a G+C content of 50% (Whiteley and Taylor, 1983). The G+C profile is illustrated in Figure 2-1, and areas in which G+C content highly diverged from this average directly relate to possible insertions (discussed below).

2.3.2 Replication and partitioning

The copy number of IncH plasmids has been estimated to be 1-2 copies per chromosome (Taylor, 1989). Plasmid replication is initiated at repeat iteron sequences after binding of a plasmid-encoded Rep protein and subsequent recruitment of host replication proteins (i.e. DnaA, helicases, primases) as the DNA helix is unwound revealing single-stranded template (del Solar *et al.*, 1998). Iteron-based replicons are indicative of theta-type replication (del Solar *et al.*, 1998), however it is unknown if uni- or bi-directional replication is initiated by any of the IncH-type replicons. R478 contains two functional iteron-based replication determinants termed RepHIA and RepHI2 (Page *et al.*, 2001). The RepHIA replicon includes the *repHIA* structural gene (SMR0001) surrounded by five upstream and nine downstream 19 bp repeated sequences (Figure 2-2). The equivalent replicons to RepHIA in R27 (RepHIA) and pHCM1 (RepA2) encode Rep initiation proteins that are 86% to the R478 RepHIA protein (Table A-1). Surrounding the R27-encoded RepHIA replication initiation protein are four upstream

Figure 2-1. Circular representation of plasmid R478 coding sequences, base composition, and comparison to other H plasmids. The outer solid ring shows the nucleotide co-ordinates, where base 1 is in the first codon of the *repHIA* gene. The second and third rings represent predicted coding sequences, in the forward and reverse reading frames, respectively. Genes are color-coded as follows: red, conjugative transfer; orange, replication; yellow, partitioning; purple, tellurite resistance; light pink, insertion elements; dark pink, chloramphenicol resistance (*cat*); brown, kanamycin resistance (*aphA*); dark green, mercuric ion resistance; grey, silver resistance; light green, copper resistance; blue, arsenic resistance; black, tetracycline resistance; light blue, miscellaneous or unknown function. The black arrowhead at base position 189001 represents the putative *ter* site. The fourth ring (blue) represents sequences that are shared between R478 and R27, whereas the fifth ring (red) represents sequences shared between R478 and pHCM1. The sixth ring (black) illustrates the G+C content variation from the overall average of 45.5%. The seventh, innermost ring (yellow and purple), represents the GC skew (see text).



and three downstream 19 bp repeats with a consensus sequence of AAAAGCATTG-ATGAATG (Newnham and Taylor, 1994). The occurrence of this replicon in all sequenced IncHI plasmids partly explains the observed incompatibility.

In contrast, the RepHI2 replicon is not present in other sequenced IncHI plasmids; however the replication initiator protein is similar to numerous other iteron-based Rep proteins (Table 2-1). A previous annotation of the RepHI2_{R478} replicon suggested that it comprised eight 18 bp iterons (four upstream and four downstream of the *repHI2* structural gene, SMR0292) and nine downstream 76 bp iterons, which each include an equivalent to the 18 bp sequence (Page *et al.*, 2001). We have redefined the iteron repeat sequences downstream of the *repHI2* gene as twelve 80-81 bp sequences that are positioned end-to-end (Figure 2-2). The previously defined four upstream repeats remain as previously described. An additional disagreement relates to the RepHI2 protein which was previously reported to be 319 aa (accession: AAB67987), whereas we predict a protein of 362 aa. The difference between these predictions occurs at the N-terminal coding region, and our prediction represents a coding sequence of similar length to the related *repA* coding sequences from pMT1 (accession: NP_395385) and pHCM2 (NP_569603). RepA_{pMT1} is predicted to be 354 aa, and displays 51% identity to the RepHI2 protein over a range of 346 aa. RepA_{pHCM2} is predicted to be 351 aa (Parkhill *et al.*, 2001), and is 51% identical to the RepHI2 protein over a range of 333 aa.

Replication termination systems in iteron-controlled plasmids are rarely observed, with the *ter*-Tus system of plasmids R6K, R1, and R100 being the only reported systems (Horiuchi and Hidaka, 1988). During uni-directional replication of R6K (or bi-directional replication of the *E. coli* chromosome), binding of the chromosomally-encoded Tus protein at the inverted *ter* repeats serves to halt the replication machinery (Lee *et al.*, 1989). In the sequence of R478 a single *ter* sequence was detected between *trhY* (SMR0206) and *trhF* (SMR0205) at base positions 188981-189001 that is nearly

Table 2-1. Predicted function of R478 coding sequences. Related sequences were determined by BLAST analysis (see Experimental Procedures).

Function	R478 Sequence	Homologues encoded in ^a
Replication	RepHIA	pMT1 (<i>Yersinia pestis</i> , pHCM2 (<i>S. typhi</i>), pSLT (<i>S. typhimurium</i>), F plasmid, pO157 (<i>Escherichia coli</i>), Rts1 (<i>Proteus vulgaris</i>), pS268a (<i>Serratia</i> sp.)
Transfer	RepHI2 TrhA-L-E-K-B-V-C-P-TraW-U-N; TrhG-H-F-Y-R-TraH-I-G-J	SXT (<i>Shigella flexneri</i>), Rts1, R391 (<i>Providencia rettgeri</i>), pRS241d (<i>Rhodobacter sphaeroides</i>), <i>Vibrio vulnificus</i> , pCAR1 (<i>Pseudomonas resinovorans</i>), pSLT, R100 (<i>S. flexneri</i>), pNL1 (<i>Sphingomonas aromaticivorans</i>), <i>Salmonella genomic island 1</i> (SGI1)
Partitioning	ParA-B	pMT1, p7, p1, pSLT, Rts1, pCP301 (<i>S. flexneri</i>)
Tellurite, phage, and colicin resistance	ParM-R	pB171 (<i>E. coli</i>), pCP301, R1 (<i>E. coli</i>), pCTX-M3 (<i>Citrobacter freundii</i>)
Organic mercury resistance	MerE-D-A-C-P-T-R	<i>Y. pestis</i> , <i>E. coli</i> O157:H7, <i>Streptomyces coelicolor</i> , <i>Deinococcus radiodurans</i> , <i>Haemophilus somnus</i> , <i>Pseudomonas syringae</i> , <i>Proteus mirabilis</i>
Silver resistance	SIIE-S-R-C-SMR0145-B-A-148-P	R100, pSB102 (from rhizosphere), pDU1358 (<i>S. marcescens</i>), R391, pKLH256 (<i>Enterobacter cloacae</i>), <i>Ralstonia metallidurans</i>
Copper resistance	CopE2-A-B-C-D-R-S-E1	pMG101 (<i>S. typhimurium</i>), <i>E. coli</i>
Arsenic resistance	ArsC-B-R-H	pRJ1004 (<i>E. coli</i>), <i>R. metallidurans</i> , <i>P. syringae</i>
Tetracycline	TetD-C-A-R	pSX267 and pI258 (<i>Staphylococcus</i> sp.), pYV (<i>Yersinia</i> sp.), <i>P. syringae</i> , R773 (<i>E. coli</i>), R46 (<i>S. typhimurium</i>)
Chloramphenicol resistance	Cat	Tn10 (R100)
Kanamycin resistance	AphA	mini-Tn10
DNA damage repair	MucA-B	Numerous bacterial genera
Sulphate permease	SfpA-B	pKM101, R391, Rts1, pCTX-M3, R46, R394 (<i>S. typhimurium</i>), R446b (<i>Morganella morganii</i>), R471a (<i>S. marcescens</i>)
Putative DNA binding proteins	SMR0042, primase; 55, Tus; 58, Cre recombinase; 60, Hha; 88, DNA helicase; 126, H-NS; 138, HigB regulator 139, OLD family endonuclease; 161, TipA; 165, cytosine methylase; 166, nuclease; 191, RelA maturase; 272, methylase; 300, adenine-methylase	Rts1, R391, Tn1404, <i>R. sphaeroides</i> , <i>R. metallidurans</i> , <i>Yersinia</i> plasmid fragment
Enzymes	SMR0037-39-40, nitric oxidase; 194, disulphide isomerase; 198, DsbC; 215, protease; 266, phosphoadenosine phosphosulphate reductase; 104/AdhC, alcohol dehydrogenase	Numerous bacterial genera

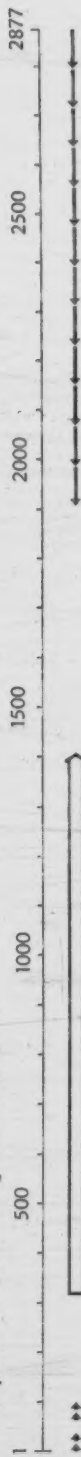
a. for references see text and Table A-1

Figure 2-2. Linear illustration of the iteron-controlled replicons of R478. Hollow arrows indicate the *rep* structural gene, while the solid arrows indicate the surrounding repeat sequences (iterons). Consensus sequences for the iterons are provided for each replicons (y = pyrimidine; r = purine; solid dash = unconserved sequence). The hatched solid line represents the nucleotide scale. The downstream RepH12 iterons, represented as twelve tandem solid arrows, include the consensus sequence present in the four upstream iterons.

RepH1A: → AAArGCATT--ATGAATG



RepH12: → TGACMAYrGTAGTGA



identical to the *ter* consensus, as well as a coding sequence, SMR0055, having ~20% identity to characterized Tus proteins (Figures 2-1 and 2-3). To my knowledge, this is the first observation of a plasmid encoding both *ter* and Tus. In the sequence of plasmids R27 and pHCM1 *ter*-like sequences were detected that were more divergent from the *ter* consensus than the R478-encoded *ter* sequence, but identical to each other (Figure 2-3). If the R478-encoded termination system is functional, a replication fork moving clockwise (in relation to the orientation shown in Figure 2-1) may be attenuated or arrested at the *ter* site, since the orientation of *ter* dictates which face of the *ter*-Tus complex is capable of blocking the approaching replication helicase (Lee *et al.*, 1989). The *repHIA* and *repHI2* structural genes are encoded on opposite strands of R478, and if uni-directional replication is initiated by both RepHIA and RepHI2 replicons, the presence of two oppositely-oriented replicons may ensure complete replication of R478 if one fork is blocked at the *ter* site. Plasmid R6K also has multiple replicons to ensure that replication of the entire plasmid is completed (Crosa, 1980).

Additionally, an asymmetric distribution of bases was observed in the R478 sequence, visualized as the GC skew, or $(G-C)/(G+C)$ (Figure 2-1). Mutational selection causes an enrichment for G and T in the leading strand (Lobry and Sueoka, 2002), and this was observed in the sequence of R478, wherein a large contiguous stretch from base positions 1600 to 166 200 of one strand has largely a positive skew, whereas a majority of the remainder of that strand has a negative skew (Figure 2-1). If the apparent inverted GC skew profile of R478 resulted from mutations favouring the replicative process, this suggests that either bi-directional replication occurs from a single replicon, as observed with circular bacterial chromosomes (Parkhill *et al.*, 2003), or that multiple uni-directional replicons are functional, each possibly creating localized GC skews.

Figure 2-3. Multiple sequence alignment of *ter* and Tus sequences. The consensus *ter* sequence (23) is aligned with putative *ter* sequences from H plasmids R478, pHCM1, and R27 (A). Known Tus proteins (*E. coli* and *S. enterica* sv. Typhi) are aligned with the putative Tus protein encoded in R478 (B). Alignments were created using ClustalW and conserved residues shaded using Genedoc. Accession numbers: *E. coli* K-12 (NP_288045); *S. enterica* sv. Typhi (CAD01897).

A. ter consensus : TTA TACAA T CTT TT
 ter R478 : TTA TACAA TT AAT TC
 ter R27 & pHCM1 : GTTAAC TACAAA TTCTTTA

B.

SMR0055 : K YDE PYFR--D I L R D S K C F S N T P P M A N I M T V S T T E E R L K Q V K P I V S T D D A S V E I I Q A T D I C V D D I E : 79
 E.coli Tus : A Y E N D T M R E I F A A H K L V A R T S L K K D E --- N I V ND S A L I Q : 80
 S.typhi Tus: Y Y E N E G D I H A A S D N S L I A S N S L A A --- A T T Y T K A A L I Q : 80

SMR0055 : S L S H S T E V T T Y P G L I I V P - E R - - A D L L L T S I N A N D A A A R R I D N K - K N V I D K V H R K L P G L V A H S T N I L F K : 157
 E.coli Tus : S N S S K P G V L L A A V H R I N K E H V E R F V H R L P G L I L L L H : 163
 S.typhi Tus: S S S K P G V L A L D N Q V R I M E Q V G R F V H R L P G L I L L L N : 163

SMR0055 : S Q L K I T S M R L N R N Q E V K A E L V L L R R E V K N V A T I N L N V V S N D A L H R L E F H E K G E S Y R I C T N S F P P A H I : 240
 E.coli Tus : - L F W H E V L A K A T R L S - - L L N K I R - - - P V V I : 239
 S.typhi Tus: - L F W E V L K S P F L S - - L Q K I R - - - P V V I : 239

SMR0055 : F A F R P E S E P N G N K Y A E T D Y S V K K S P I F A A G N I F Q L T S E W A P E S S G P S N Q R K L S L Y T E V F G A E C H F I V S P E N : 320
 E.coli Tus : P C H - - - I A A I R V A V E L A Y N S L S - - I I R - - L L Y V - - - : 309
 S.typhi Tus: P C H - - - S A A T V R G E W I S L S - - L S - - I I R - - L L Y V - - - : 309

Active partitioning of R27 is a result of two distinct modules (Par1: *parSAB* and Par2: *parCMR*) inserted among the transfer genes, each capable of supporting partitioning, and Par1 being the major determinant (Lawley and Taylor, 2003). Each module is comprised of an array of direct repeat sequences (*parS* or *parC*) which serve as the centromere region for binding by ParB or ParR proteins, with which the respective ATPases (ParA or ParM) interact (Lawley and Taylor, 2003). R478 contains a 3080 bp region preceding the predicted *parA* and *parB* genes (SMR0014/15) that includes 11 repeats ranging from 233-257 bp, which are termed *parS* (Figure 2-4). Additionally, each of the 11 repeats contains a highly conserved sub-region (ATTTAACACCCTTAAT CGCCAAACTGG) that aligns with the 26 direct repeats (34 bp with consensus: cccCctTAaTcGcCag--ccATGG-gg-a--c-g) present in the R27 *parS* region. Analysis of R27 *parS* outside of the 34 bp repeats did not reveal any additional conservation with the remainder of the larger R478 *parS* repeats.

A region similar to the second R27 partitioning module, Par2, was also detected in the R478 sequence (SMR0020/21; Figure 2-4). The ParM ATPase of this module is predicted to be an actin-type ATPase, rather than a Walker-type ATPase such as ParA encoded in the Par1 module. The nucleating proteins (ParR) of actin-type partitioning modules are less conserved, however the genetic organization of the Par2 structural genes of both R27 and R478 is conserved. Repeat sequences which could act as a centromere region in the Par2 region of R478 were not detected. The dual partitioning modules encoded on R478 resemble numerous plasmid-encoded sequences (Table 2-1). The presence of multiple replicons and partitioning modules may be a result of multiple evolutionary events leading to the duplication of these core plasmid systems, with selection and maintenance of each module ensuring the continuance of the plasmid in a broad range of hosts.

Figure 2-4. Linear illustration of sequences encoding conjugative transfer functions. Individual coding sequences are represented as coloured arrows, where solid colored arrows represent sequences homologous to the same-coloured essential R27 transfer gene; hollow coloured arrows represent sequences homologous to the same-coloured non-essential R27 gene; hollow black arrows represent unique sequences, and black solid arrows represent repeat sequences found in the partitioning and origin of transfer (*oriT*) regions. On the left, the source of the sequence is listed and the prefix for the nomenclature of the genes is in parentheses (example: the R478 gene 'A' represents '*trhA*', whereas R478 genes not having a *trh* prefix are labeled in their entirety). Solid lines above the arrows indicate contiguous sequences, and the R478 sequence is labeled with a nucleotide scale. Genes are transcribed in the direction indicated by the respective arrow. Citations: pCAR1, (Maeda *et al.*, 2003); R27, (Lawley *et al.*, 2002a; Lawley *et al.*, 2003a); SXT, (Beaber *et al.*, 2002); R391, (Boltner *et al.*, 2002); Rts1, (Murata *et al.*, 2002); pNL1, (Romine *et al.*, 1999)

Within the R478 sequence additional sequences which may be involved in plasmid segregation were detected. SMR0243a displays 69% amino acid identity with Hok, a 52 amino acid peptide encoded on plasmid R1 which acts in post-segregational killing through its toxic effects to membranes (Gerdes *et al.*, 1997). Synthesis of Hok is prevented by antisense *sok* RNA, and only daughter cells receiving the plasmid (and thereby the ability to produce *sok*) will survive, ensuring the maintenance of the Hok/*sok*-carrying plasmid. This module in R478 is surrounded on either side by insertion sequences (*insLKJ*), and was likely to have been acquired as a cassette. SMR0278 (92 aa) is also related to a killer peptide: the 92 aa HigB of plasmid Rts1, shown to have post-segregational killing activity (Tian *et al.*, 1996). However, an equivalent to the suppressor protein HigA could not be detected, and it is likely that because of the modest level of conservation between the R478 and Rts1 HigB proteins (30% identity), that R478 HigB has an alternative function. The implicit genetic burden on the host cell by the large size of R478 may have resulted in the presence of multiple partitioning and killing functions, thereby decreasing the chance for elimination of R478 after segregation.

2.3.3 Conjugative transfer

One of the defining phenotypes of IncHI plasmids is the ability to transfer between cells via bacterial conjugation. Conjugation is a paradigm for horizontal gene transfer, and facilitates the spread of various genetic determinants throughout bacterial populations. This process is presumed to be initiated by the H-pilus, an extracellular filament comprised solely of pilin subunits (Lawley *et al.*, 2003b). The H-pilus is responsible for contact with recipient cells, then retracting to allow the transfer pore to form between the two bacterial cell walls. The transmembrane apparatus (the Mpf complex) that is required for pilus production, is also considered to be involved in pore

formation and the subsequent translocation of DNA. Plasmids are transferred to recipient cells as single-stranded DNA, a product generated by the cytoplasmic relaxosome complex, which includes DNA-binding proteins and a relaxase responsible for DNA cleavage at the origin of transfer (*oriT*). The Mpf complex and relaxosome are linked via an integral inner membrane coupling protein, completing the conjugative apparatus.

The transfer genes of R27 and pHCM1 are present as two separate regions, termed Tra1 and Tra2. A majority of the Mpf proteins are encoded in Tra2, whereas the *oriT*, some Mpf genes, and all relaxosomal components are encoded in Tra1 (Lawley *et al.*, 2002a; Lawley *et al.*, 2003a). The same transfer gene organization occurs in R478 and the individual gene products are 45-97% identical to the respective R27 transfer proteins (Figure 2-4). Essential R27 transfer proteins were previously determined in large-scale mutagenesis studies (Lawley *et al.*, 2002a; Lawley *et al.*, 2003a). In comparison with proteins encoded in the R27 transfer system, but which are not essential for R27 transfer, the respective R478 transfer proteins have an average percent identity of 64% (n=12; range: 45 – 85%), whereas essential transfer proteins are more conserved (average: 80%; n=21; range: 63 – 97%). The high level of both sequence and organizational conservation of the R27 and R478 transfer sequences implies that they share a common ancestor; however, mutations have accumulated in both essential and non-essential genes, with the greater occurrence in the later. The majority of the R478 transfer genes have been renamed with the prefix *trh*, as previously used for both R27 and pHCM1 transfer determinants, in preference to the prefix *htd* initiated by Page and colleagues (Page *et al.*, 1999).

The Mpf genes of all three sequenced H plasmids resemble those encoded on the genetic elements SXT, R391, Rts1, pNL1, and pCAR1 (Figure 2-4 and Table A-1). Each of these transfer systems resemble the prototypical transfer determinants of the F

plasmid in regards to both sequence identity and genetic organization. During the search of the genome databases for transfer sequences related to R478, two novel sequences having the same organization as the H plasmid transfer system were found. The genome data available for *Rhodobacter sphaeroides* 2.4.1 (accession: NZ_AAAE01000159) and *Vibrio vulnificus* CMCP6 (NC_004460) each contained putative T4S systems which encode an equivalent to each of the essential H plasmid Mpf transfer genes, but lack the inserted elements such as the partitioning modules and many of the non-essential transfer genes of H plasmids (Figure 2-4). Also, both the *R. sphaeroides* and *V. vulnificus* Mpf-like sequences are encoded in one contiguous segment, rather than in separate Tra regions, as observed in H plasmids. These novel sequences may represent a prototype of the H plasmid Mpf transfer systems since they each lack insertions (i.e. par modules) and non-essential Mpf genes. *R. sphaeroides* 2.4.1 contains a 100 kbp conjugative plasmid identified as pRS241d, and the R478-like transfer sequences were localized to this element (S. Kaplan and R.C. Mackenzie, personal communication). The *V. vulnificus* transfer genes were localized to chromosome II, as reported in the available submission. It is possible that the *Vibrio* genes are not involved in conjugative transfer, but rather T4S of an unknown substrate.

The relaxosomal (Tral and TraJ) and coupling proteins (TraG) of the H plasmids, SXT, R391, Rts1, and pCAR1 are not strict members of the F-type family, and constitute a novel phylogenetic group (Figures 2-5, 2-6, and 2-7). For each of Tral, TraG, TraJ, and the respective related proteins there is conservation of both primary amino acid sequence (including highly conserved subdomains), and predicted secondary elements, such as transmembrane domains (Figures 2-5, 2-6, and 2-7). The genomic context of *tral*, *traG*, and *traJ* is also conserved in multiple genetic elements, including the above sequences, plasmid pHG1, and the genomic sequences of *Haemophilus somnus*, *Xanthomonas axonopodis* pv. *citri*, *Pseudomonas fluorescens*,

2

Figure 2-5. Multiple sequence alignment of a conserved region from IncH Tral-related sequences (A). The alignment was generated using ClustalW in MegAlign (DNASTar) and conserved residues were shaded using GENEDOC. Previously characterized relaxase motifs containing conserved His and Ser residues are indicated with a '2' (see text), whereas novel conserved regions of the IncH-type Tral proteins are indicated with a '1'. Possible nucleophilic tyrosine residues are circled. Accession numbers: R478 (CAE51736); R27 (NP_058333); SXT (AAL59675); R391 (AAM08003); pCAR1 (NP_758664); Rts1 (NP_640161); *Haemophilus somnus* (Hso, ZP_00123099); *Pseudomonas aeruginosa* (Pae, AAN62266); *Xanthomonas axonopodis* pv. *citri* (Xac, NP_642513); *Pseudomonas fluorescens* (Pfl, ZP_00087872); *Ralstonia metallidurans* (Rme, ZP_00023889); *Burkholderia fungorum* (Bfu, ZP_00030759). Rooted phylogenetic tree generated by MegAlign using the multiple alignment of the complete sequences for each of the above Tral homologues (B).

A.

2 1a

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TraI R478 : RPK HSTSMMLA DGDIREDDAYT TNIVLKPVEQVRW HLPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 164
TraI R27 : RPK HSTSMMLA DGDIREDDAYT TNIVLKPVEQVRW HLPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 162
TraI SXT : DEL AKLRQVIGFNLR-----D QRLIQPCIDHL AQ HLPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 118
TraI R391 : DEL AKLRQVIGFNLR-----D QRLIQPCIDHL AQ HLPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 118
TraI pCAR1 : HMLFDR LHSVG PAS-----DLKIQVEPIVN ELAHMHPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 197
201 Rts1 : ERT SKKREIM RDA-----E DEIQPMITN NF HLPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 134
1101 Hso : QHH GLN QVS TRE-----M EHLQKHEP EM CLHPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 123
SG44 Pac : QRL EHWRTS SRQ-----C TALYLAKP EO SI QHPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 116
2196 Xac : QRL EHWRTS SRQ-----C DALYLAKP EO SI QHPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 124
5158 Pfl : ROL DRWCTA SHV-----Q TOFYLNKTER EC QHPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 112
2852 Rme : QRL EHWRTS SRK-----Q AVLYRAKTER EL QHPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 403
3583 Bfu : QRL EHWRTS SRK-----Q ATLYRAKTER EL QHPASENHHHNGIG LLSSELEVAILSLNNAHSEIRPI : 116
  
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1b

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TraI R478 : GYQDEVVRRKVVLY AFICGLHAGKYDLOTVSNLSPITWTPSSQSLLEARENDDVEYEIHWKKRIHQHNIW : 244
TraI R27 : GYQDEVVRRKVVLY AFICGLHAGKYDLOTVSNLSPITWTPSSQSLLEARENDDVEYEIHWKKRIHQHNIW : 242
TraI SXT : GTVEKKELEPRMV AALGGLEHEDGHP-VSDSITDEI RYQWNPFLTELSCWTTNNGIER YRWDGRCRKEQFS : 197
TraI R391 : GTVEKKELEPRMV AALGGLEHEDGHP-VSDSITDEI RYQWNPFLTELSCWTTNNGIER YRWDGRCRKEQFS : 197
TraI pCAR1 : ETARRSMRLRRVY GIASGLEHEDGHP-LTDRTATDFE NNQWYIAQETLHWSVRNKLTRY LHMNSNRHGNVQVS : 276
201 Rts1 : EGVIKSDRALMRI VVVGALHEDGHP-ISDVCWDKS EKHWPFAVHCLHFAETKIER YLWSDRHERHNTS : 213
1101 Hso : AAEDAKQKDMTA VIYLAHEDGHP-INDTELOLD K-----RWLAHGIPTLPYK RYHQDDEYRLSAAT : 194
SG44 Pac : TTEQVIAQAEAMTA VAYAAHEDGHP-AVDHVEYGI S-----QWHPHGLPSQPYR RYRDEYRLSAAT : 187
2196 Xac : ATEQAAQAEAMTAGVAYAAHEDGHP-AVDHVEYGI S-----AWRAHGLVLPQYR RYRDEYRLSAAT : 195
5158 Pfl : AAEDAAQTDAASAGIAYGALHEDGHP-AVDHVEYGI R-----VWHPHGLSQQPYR RYRDEYRLSAAT : 183
2852 Rme : ATEQAAQAEAMTA VAYAAHEDGHP-AVDHVELAD S-----LWHPHGLHQPQYR RYRDEYRLSAAT : 474
3583 Bfu : STEQAAQAEAMTA VAYAAHEDGHP-AVDHVELAD S-----TWHPHGLHQPQYR RYRDEYRLSAAT : 187
  
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TraI R478 : SVFLERILNPVC AFLDRVNKERY SKMTALNVYTDGND-----FUSKVRTEFYTGTLHVLADPIMG : 311
TraI R27 : SVFLERILNPVC AFLDRVNKERY AKMTALNVYNDGND-----FUSKVRTEFYTGTLHVLADPIMG : 309
TraI SXT : ILVNLNVYTPPEL AMLTOPGPE-ILQAEELIGNTDPEH-----FUSKVRTEFYTGTLHVLADPIMG : 261
TraI R391 : ILVNLNVYTPPEL AMLTOPGPE-ILQAEELIGNTDPEH-----FUSKVRTEFYTGTLHVLADPIMG : 261
TraI pCAR1 : VAQATRIEPPAVQMLIEGGHD- EALDMSGGSS-----PTEFVKKWTSATKRLNRGSGNNGG : 339
201 Rts1 : LTKMTIIPKSI AFIMQEGND- NEITEFASNSFRAVTSRNETGTGVFNKHKH AHASRVEKCHLQRYSGDAVR : 292
1101 Hso : GFIANQLHAKETFDHAT-YEP-SALYYVAGHYEKAN-----VLAEPVOKRONEVALAGGDDITKLV : 258
SG44 Pac : GLYRQVLADRI DMLR-YFP-SALYYVAGQYEHAG-----TGEVWQIRASVAGQGGDPARAM : 251
2196 Xac : GLYARVLTGIDMLR-G-FPP-SPIFYVAGQNEHAG-----TGEVWQIRASVAGQGGDPARAM : 259
5158 Pfl : GLYATQILARPIDMLR-G-FPP-ASLYVAGQYERAG-----TGEVWQIRASVAGQGGDPARAM : 247
2852 Rme : GLYRQLITQIDMLR-D-YRD-LGPLYYVAGQYEHAG-----TGEVWQIRASVAGQGGDPARAM : 539
3583 Bfu : GLYRQLITQIDMLR-G-YPA-LGPLYYVAGQYEHAG-----TGEVWQIRASVAGQGGDPARAM : 261
  
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TraI R478 : --IRSNDAAALA STI HNFTSININNYNAKPMHIIVNG--EVLNENAFLDVFLNDFELHKYN-FQGEAGKTVIVE : 385
TraI R27 : --IRSNDAAALA GTI HNFTSININNYTKPMHIIVNG--EVLNENAFLDVFLSDFAAHKFN-FQGGDAGKTVIVE : 383
TraI SXT : DNALGVPEVRY DAMR ASSQWLVNQDARV LRKSNQSTHLYL WKAAKDIHEL AKDKIPG-----RDPDTIAD : 338
TraI R391 : DNALGVPEVRY DAMR ASSQWLVNQDARV LRKSNQSTHLYL WKAAKDIHEL AKDKIPG-----RDPDTIAD : 338
TraI pCAR1 : GNATGVPEVRL SDAMLR SDGTWKNITPGGVR VATEG-----YVWNOGAEEIVSMVGDGVVA-----RSPDTIIG : 412
201 Rts1 : ASQTGVPEVIR DAML KKEEWKPNQAGSFL VATEG-----VFIWGTAVTEVSIK-ESGINF-----HSADSIAD : 364
1101 Hso : -QKPVISFAQD LALY SQKFKISSKPGGDC LTEDG-----LWL SKTVSDKRAH LSGQIDG-----SSNITAFD : 329
SG44 Pac : -AAPKHALQK LALY KEELKINQPGASDG LTEDA-----LWL SKTVSDKRAH LSGQIDG-----ANNTAFD : 323
2196 Xac : -TAPRHALQK LALY REQLQINQPGASDG LTEDA-----LWL SKTVSDKRAH LSGQIDG-----SSNITAFD : 331
5158 Pfl : -QAPIHSLQHH LALY QHELKINQPGAGG LTEDA-----LWL SKTVSDKRAH LSGQIDG-----SSNITAFD : 318
2852 Rme : -AAPKHALQK LALY KEELKINQPGASDG LTEDA-----LWL SKTVSDKRAH LSGQIDG-----ANNTAFD : 610
3583 Bfu : -AAPKHALQK LALY KEELKINQPGASDG LTEDA-----LWL SKTVSDKRAH LSGQIDG-----ANNTAFD : 323
  
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TraI R478 : SLVQRYYKPYDDEVVHYFIPGIYSENEISNIFRNGIGKLEFYNI ERWIGIFDSYKIFDSVP-LFSVNANKDFIY : 464
TraI R27 : SLVQRYYKPYDDEVVHYFIPGIYSENEISNIFRNGIGKLEFYNI ERWIGIFDSYKIFDSVP-LFSVNANKDFIY : 462
TraI SXT : LIER ATKPASNERYESLAPEVLI-----KDDK-P-IWLPMLHSEADLFSSN-VSSSVR----- : 393
TraI R391 : LIER ATKPASNERYESLAPEVLI-----KDDK-P-IWLPMLHSEADLFSSN-VSSSVR----- : 393
TraI pCAR1 : SLVQR AKRAPNDLYGLVTPHLLR-----KNGKGP-ALRCMVNPDLEFPTTIPFVS-----IG : 470
201 Rts1 : LNLNY CVLNSDVSYYRLAPHLNDK-----ISQKKEPKNALSGVVDPLFTDDVMNETSCRIKLADGWKEFTG : 440
1101 Hso : EQQARR IHSSENAWYQQLSADA-----GWKPK-DKFSILHKEPEWDNIDDRBELFA----- : 386
SG44 Pac : VLQDH LQPTPGKAIWRATVTSTA-----GWA-----HTFTLAPALWE-GENRDEFTT----- : 377
2196 Xac : VLQDH LALPDEGKAIWRACVASES-----GWS-----HTFTLAPALWE-AGERAPFA----- : 385
5158 Pfl : ELQSH VESTPEGKAIWALVAQG-----NWQ-----QSTELAPALWE-SDNRTEVFS----- : 371
2852 Rme : VLQDH LHPITIMKAVARATVTSNA-----GWT-----HSFTLAPALWE-AGERAPFA----- : 664
3583 Bfu : VLQDH LHPITIMKAVARATVTSAT-----GWS-----HSFTLAPALWE-PGERVVFPA----- : 377
  
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B.

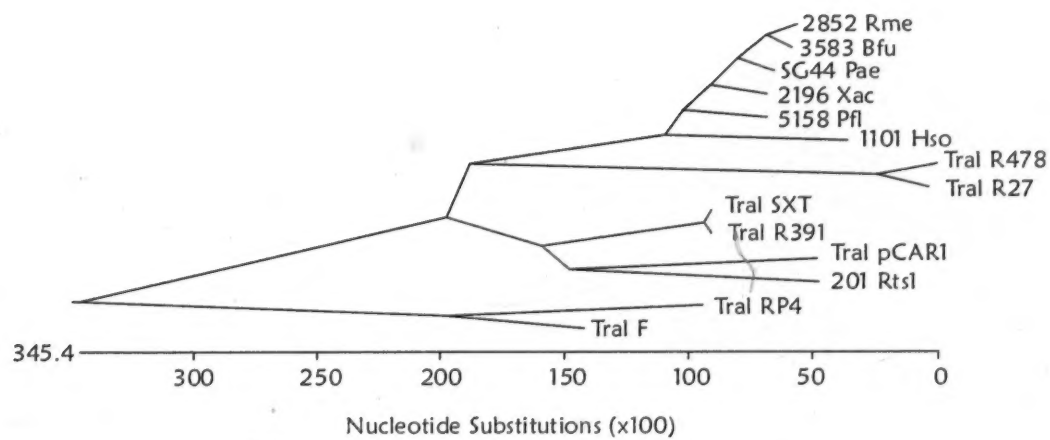


Figure 2-6. Multiple sequence alignment of TraG-related sequences (A). The alignment was generated using ClustalW in MegAlign (DNASStar) and conserved residues were shaded using GENEDOC. Underlined residues indicate transmembrane helices predicted using TMHMM, boxed regions are predicted periplasmic domains, and Walker NTP-binding motifs are indicated. Accession numbers: R478 (CAE51737); R27 (NP_058332); SXT (AAL59680); R391 (AAM08004); *Ralsotonia metallidurans* (Rme 5679, ZP_00026653); pHG1 (AAP86115); Rts1 (NP_640162); pCAR1 (NP_758665); *Salmonella enterica* sv. Typhimurium (Sty, NP_458648); *Haemophilus somnus* (Hso, ZP_00123135); pWWO (NP_542873); *Pseudomonas aeruginosa* (Pae, AAN62290); *Burkholderia fungorum* (Bfu, ZP_00030778); *R. metallidurans* (Rme 4237, ZP_00025244); *Pseudomonas fluorescens* (Pfl, ZP-00087890); *Xanthomonas axonopodis* pv. citri (Xac, NP_642576); *Pseudomonas syringae* (Psy, ZP_00127721). Rooted phylogenetic tree generated by MegAlign using the multiple alignment of the complete sequences for each of the above TraG homologues (B).

A.

TraG R478 : MSDSKRTNLHACINFY P1 YRSASILLICSVSN YMGSSGKLLIAPIVLFTSILLFLLCLYRCKTAAP MAHNR P : 81
 TraG R27 : MTKSKRTNLHACINFY P1 YRSASVLLICVLE VMGFFRQVNIAPILLYTAVFI LVCYRCKTAHPY MAHNR P : 81
 TraD SXT : -----MPK TNY AMAAGWLVG TGA AEML-----TELP EPFWMTCIGSGMALYNPEAYR Y : 59
 TraD R391 : -----MKNAYEMPW TNY AMAAGWLVG TGA AEML-----TELP EPFWMTCIGSGMALYNPEAYR Y : 66
 5679 Rme : -----MMNHYNHF PTY FRAAVGWVAGL I LSGMPH-----AGYFALLC CALLRRAQVWRAKFRLA ST : 66
 TraG pHG1 : -----MNQYINHF PTY FRAVVGWALAVLI L VSGMPN-----SGYFALGGAFLLFRSSQVWRAKFRLS ST : 65
 202 Rts1 : -----MSEWKYNMPW INY AFEVAGWSAGTASS AVNAF-----FDMPLPCYMAVAVQAAAYGTRATPAYR Y : 66
 TraG pCAR1 : -----MSEWDYTSYK KNY AREAVAWGSSMLAGTAVOLI-----TCIVTPYIITLGVQAGFALAA PRALR H : 66
 4562 Sty : -----MSNRYVMALL PAV LYSAAGGAATF L VAPWS-----MALAIQVSYVTAAGFAIFTTITBQGLR LR : 67
 1138 Hso : -----MASKYLLEGIL PP FYPAAHAACAYICAFAPWS-----LANMIGYGLAASFSGISYL FQGWPR V : 67
 106 pWNO : -----MAQPHAVVILL PA LYTVAVCTGA I C VAPWS-----LANMLG GSALATLTFGAILADAWA LR : 67
 SG68 Pae : -----MAQPHAILVLL PA LYTVAVCAAAAL S VAPWS-----LALSTAG GSALATLTFGAILADALV LR : 67
 3602 Bfu : -----MSGKQPVVILL PAV LYTVACAGAA F S VAPWS-----LALSTAMG GSALAFGAYGAIKYDARV LR : 67
 4237 Rme : -----MSGKQPVVILL PAV LYTVACAGAA F S VAPWS-----LALSTAMG GSALAFGAYGAIKYDARV LR : 67
 5177 Pfl : -----MAENAMESKL PA LYTVATCTAA V C YBERA-----VALSIEG VVALAYALFGLITBQWE LR : 66
 2259 Xac : -----MAHDYATISLL PAV LYTVYVCAACAFIC PAVPA-----FALITLFG VTSAGFLALGLVPRCAWQ LR : 67
 4044 Psy : -----MAHDYATISLL PAV LYTVYVCAACAFIC PAVPA-----FALITLFG VTSAGFLALGLVPRCAWQ LR : 67

TraG R478 : RHMFVSLDSLRVINKSNFFSNERKYRLVQDYONAKL PERKS FCD E GPE ADRAVQIANLSSDKREIELPFFVN : 162
 TraG R27 : RQIMFISLRLTINKSNFFSNERKYRLVQDYONAKL PERKS FCD E GPE ADRAVQIANLSSDKREIELPFFVN : 162
 TraD SXT : LQMG YGK-LAFM-----ALSHLQVIAKHPEDEL Y E DPE ADRAVQIANLSSDKREIELPFFVN : 119
 TraD R391 : LQMG YGK-LAFM-----ALSHLQVIAKHPEDEL Y E DPE ADRAVQIANLSSDKREIELPFFVN : 126
 5679 Rme : KWLTVVLD-DLL-----DASRGISLSDKDDVDAN T E TQK CULANDIL--RMPSTDIPLPKWL : 128
 TraG pHG1 : KWLTVVLD-DLL-----DASRGISLSDKDDVDAN T E TQK CULANDIL--RMPSTDIPLPKWL : 127
 202 Rts1 : HKR AKKHVDLIM-----RIEQIDAMIKYPPDCYIF K Q GNEGSAFILL--RDIKKLTAKT-- : 127
 TraG pCAR1 : AKAC VGAD-LSFI-----TLEEVIELKRRHPEF EA P RQK GUYAFELQ--AMDIEAI KGRKR : 129
 4562 Sty : YRN RRLP-----RYQMSSEIPVSRHF K M EEP TALLTK--PEVAPFLKPSVFY : 126
 1138 Hso : YRN RRLP-----HYALTSIC/PVNNKFL L R E QPE TALLYDCE--SADGKYLRLPSKLF : 126
 106 pWNO : YRN RRLP-----RYVMTSLDVPVSOQR V P R ECH THLMJTY--PEFERRY EPTAY : 126
 SG68 Pae : YRN RRLP-----RYVMTSLDVPVSOQR R R ECH THLMJTY--PEFERRY EPTAY : 126
 3602 Bfu : YRN RRLP-----RYVMTSLDVPVSOQR R L ECH THLMJTY--PEFERRY EPTAY : 126
 4237 Rme : YRN RRLP-----RYVMTSLDVPVSOQR R L ECH THLMJTY--PEFERRY EPTAY : 126
 5177 Pfl : YRN RRLP-----RYGLTSIQIPVSKRL R R TRL TRLVLAQ--PPAVAHY EQPTRY : 125
 2259 Xac : YRN RRLP-----HYTMTSEI/PVSNQRL L R QCH TRLMDTY--LPKYASYEATSLF : 126
 4044 Psy : YRN RRLP-----HYTMTSEI/PVSNQRL L R QCH TRLMDTY--LPKYASYEATSLF : 126

Walker A

TraG R478 : PIKHHFDA-----MARKHESNAFAARRP--FUTEDNWF H ITNVGTGTVIQ : 215
 TraG R27 : PIKHHFET-----MARSHENNAFAARRAP--FUTEDNWF H ITNVGTGTVIQ : 215
 TraD SXT : -----HGKQMSTW H PKREY YQPUGHT H GAGHT CF : 164
 TraD R391 : -----HGKQMSTW H PKREY YQPUGHT H GAGHT CF : 171
 5679 Rme : PNKTVRAIEKAFAP-----A-----NSIADHSPCKPWIA L TKKOP PFHYKAMG H SISC GAGHT TF : 192
 TraG pHG1 : PKTVKAIETKAP-----E-----NSIADHSPCKPWIA L SLRIP PFHYKAMG H SISC GAGHT TF : 191
 202 Rts1 : -----DDDFMLGWI Q GSDNDY RTVENLLSLIT GSKHTTY : 172
 TraG pCAR1 : -----NAE KTKK H GMDIEEPCTRPADORSIH V GAGHT WF : 175
 4562 Sty : SLAQFERRIYET--FPGCLSLTRDSFFNPVRPLPT ENSA H MEEDN LLSITERY H V GAGHT FA : 205
 1138 Hso : NMAIEYKHHENI-----FTKLTTSDSCFNVRPLPP EIPAG H LNEID MCHNSAC H V GAGHT FA : 200
 106 pWNO : RAA RLEERLEFAPFPVSKLARALAWDSPLNPARPLP LPR H PNEVJ TILGERV H V GAGHT FA : 206
 SG68 Pae : QLA RLEERLEFAPFPVSKLARALAWDSPLNPARPLP LPR H PNEVJ TILGERV H V GAGHT FA : 206
 3602 Bfu : RLTRLEERLEFAPFPVSKLARALAWDSPLNPARPLP LPR H PNEVJ TILGERV H V GAGHT FA : 206
 4237 Rme : RLTRLEERLEFAPFPVSKLARALAWDSPLNPARPLP LPR H PDEVJ TILGERV H V GAGHT FA : 206
 5177 Pfl : RLALGLERRLEHAPFPFETLARATWDSAPNPLRPLP VESPL H PDETE TILGERV H V GAGHT FA : 205
 2259 Xac : -----YRPLFFVGLPH H PEEMJ TILGERV H V GAGHT FA : 48
 4044 Psy : RAA RFEERAEPFAPYVRLARATSWDVPINVRPLPP LPR H PYFEN TILGERV H V GAGHT FA : 206

TraG R478 : RLSISMHLG-----HYVADPPKNAEWRESMEBAKTL LP--YK PQAASVCI VCNTITNV DITRLSL : 288
 TraG R27 : RLSISMHLG-----HYVADPPKNAEWRESMEBAKTL LP--YK PQAASVCI VCNTITNV DITRLSL : 288
 TraD SXT : DAM A LKN-----LA IDPKK KEIKDNAQRACIA SPER VYS PQAASV L P RNENRG LA KPAAL : 239
 TraD R391 : DAM A LKN-----LA IDPKK KEIKDNAQRACIA SPER VYS PQAASV L P RNENRG LA KPAAL : 246
 5679 Rme : EIS V HNP-----NDV IDPPKNAEWRESMEBAKTL LP--YK PQAASV L P RNENRG LA KPAAL : 266
 TraG pHG1 : EIS V HNP-----NDV IDPPKNAEWRESMEBAKTL LP--YK PQAASV L P RNENRG LA KPAAL : 265
 202 Rts1 : K ILL AALR-----LP IDPPKNAEWRESMEBAKTL LP--YK PQAASV L P RNENRG LA KPAAL : 247
 TraG pCAR1 : DAI C AFG-----LP IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 250
 4562 Sty : L L D RKG-----NVT IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 278
 1138 Hso : K LV D HRGKTOAERV IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 281
 106 pWNO : K LV D RKKVRGEHFA IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 286
 SG68 Pae : K LV D RARNAAGEHFA IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 287
 3602 Bfu : K LV D RARNAAGEHFA IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 287
 4237 Rme : K LV D RARNAAGEHFA IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 287
 5177 Pfl : K LV D H I-----EHEV IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 282
 2259 Xac : K LV D RRAADGGHEV IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 129
 4044 Psy : K LV D RARNAAGEHFA IDPKK KGLAETCERCERAV KASL KY PARCKOV LSLTKN KA VAVVVL : 286

TraG R478 : --VP-EVNRVVA KALCN ISS SYEK GIIYLHN KS--HMSIVNLTIVKVMSCYAY GYD WTEKVYVAN : 366
 TraG R27 : --VP-EVNRVVA EALGT ITG SYTDK GIIYLHN KS--HMSIVNLTIVKVMSCYAH GPD WMEKVYVAN : 366
 TraD SXT : --P-SGTGADPTFA GOMAN N VQC LTSQ PUKTREF EG---GPEGLVVKAVTANGEQV PN--FS-----VEIK : 308
 TraD R391 : --P-SGTGADPTFA GOMAN N VQC LTSQ PUKTREF EG---GPEGLVVKAVTANGEQV PN--FS-----VEIK : 312
 5679 Rme : --E-----EGPTF LPL RAVAGE YVMD NLRSLQYAG GINN LERLOR PEAGTTE KDF AG-----YMQ : 335
 TraG pHG1 : --E-----EGPTF LPL RAVAGE YVMD NLRSLQYAG GINN LERLOR PEAGTTE EEEAAG-----YMQ : 335
 202 Rts1 : --QR-ISTSDPTIS TMS TS LEM KMCKI TILKSXQ GS-NITALARLVQAITKVRGVI.PHG-IDS-VLAGKKS : 326
 TraG pCAR1 : --K-SIAG-DPFOAT OMS S ICA ICNII TLVGR RT EG---DVPGLTIVKVMAPGSPV GEDEFAR-M--ADSA : 322
 4562 Sty : --G-SINNAAEYEE WRRI I ARA VLRL PYSILLY TH-IAEYETIAEKLITQCAPEI LMVQNM-GILTED : 356
 1138 Hso : --G-SINNAAEYEE WRRI I ARA EYGE PYSILLY TH-IDSIFLNYAKTIPDARDTAL POLITIA-AGVDVKN : 359
 106 pWVO : --G-SINNAAEYEE WRRI I ARA ELQO PYSILLY TH-IDAIEYAOH PARNBKA EVI QLE-AKLNDK : 364
 3602 Bfu : --G-SINNAAEYEE WRRI I ARA ELQO PYSILLY TH-IDAIEYAOH PAKTEFA EVI QLE-AKLNDK : 365
 4237 Rme : --G-SINNAAEYEE WRRI I ARA ELQO PYSILLY TH-IDAIEYAOH PAKTEFA EVI QLE-AKLNDK : 365
 5177 Pfl : --G-SINNAAEYEE WRRI I ARA ELQO PYSILLY TH-IDAIEYAOH PAKTEFA EVI QLE-AKLNDK : 360
 2259 Xac : --G-SINNAAEYEE WRRI I ARA ELQO PYSILLY TH-IDAIEYAOH PAKTEFA EVI QLE-AKLNDK : 207
 4044 Psy : --G-SINNAAEYEE WRRI I ARA ALAR PYSILLY TH-EGTPOEYASKVPEYDEFA EAI AIE-GKLNDK : 364

TraG R478 : TLPVRFK LAEWFTAHFMN EGSEQ--IDWLDIVSO IDYSMS PEHMAPT GHPVDM IEKP NLSHPNSVS-- : 443
 TraG R27 : TLPVRFK LIEWFNHFLN EGAEF--IENDTGR VDYMS PEHMAPT GHPVDM IEKP NLSHPNSVS-- : 443
 TraD SXT : --PTEKANTIAKQAMALF YERIQP-VAAPTDE L MFEH TH SEM GHPV NML SEIGPLLSIAN /D-- : 386
 TraD R391 : --PTEKANTIAKQAMALF YERIQP-VAAPTDE L MFEH TH SEM GHPV NML SEIGPLLSIAN /N-- : 393
 5679 Rme : --QVGKG GT--RLDAM RL IDRFSLKNGHEA I TYOH DH QRI ALPL JMA SETGLHAKRAD FE-- : 413
 TraG pHG1 : --KVAQKA GT--RLDAM RL IDRFSLKNGHEA I TYOH DH QRI ALPL JMA SETGLHAKRAD FE-- : 412
 202 Rts1 : --RMSGVVDSVSIKCAEI HDIRH-NKAFPEAGIDTSH EH QRI LN MFI SOL FNGDLSSTY PDQM : 406
 TraG pCAR1 : --ILSAKVRNTEKAKVLRKV YNDIAK-VEANTDE L MFEH TH SEM GHPV NML SEIGPLLSIAN /D-- : 399
 4562 Sty : --PRTMO QVNAIRVMAIEVA LSSQGGKIVNVL R AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 435
 1138 Hso : --LSPGMDP---LIWVINO ILENK---IPNVLE A AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 432
 106 pWVO : --IPNMIGRE--KRVALEQ LSOVR---VYVVL R AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 438
 3602 Bfu : --IPNMIGRE--KRVALEQ LSOVR---VYVVL R AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 439
 4237 Rme : --IPNMIGRE--KRVALEQ LSOVR---VYVVL R AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 439
 5177 Pfl : --IPNMIGRE--KRVALEQ LSVKR---VYVVL R AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 434
 2259 Xac : --IPNMIGRE--KRVALEQ LSOVR---VYVVL R AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 281
 4044 Psy : --VFNMKIRP--FRVAIDQ LSOVR---VYVVL R AVRY TY DKT ALPL JMA SETGLHAKRAD FE-- : 438

Walker B

TraG R478 : --SREVTSDGPFSTGGY YIS GHPES R SQL MSOLT CA SRNADDGJ-----MSANSHGTFVMAHS : 514
 TraG R27 : --SREVTSDGPFSTGGY YIS GHPES R SQL MSOLT CA SRNADDGJ-----MSANSHGTFVMAHS : 514
 TraD SXT : --SRLIT SGRINNAOAYIT RSLTAM GS LSL LSOLTA DRNYGVEN-----RNVHEDTAA : 454
 TraD R391 : --SRLIT SGRINNAOAYIT RSLTAM GS LSL LSOLTA DRNYGVEN-----RNVHEDTAA : 461
 5679 Rme : --DREIA IDKV KCAVYIT ASLSNM Q MSM LADIA TA AINNYAKP-----PEMVVDDAA : 481
 TraG pHG1 : --DREIA IDKV KCAVYIT ASLSNM Q MSM LADIA TA AINNYAKP-----PEMVVDDAA : 480
 202 Rts1 : --E DRIA VREIT STNGVYL AAGDGT A LSLRSLT VASEIN MOPRGD-----I-ANILIDEAA : 479
 TraG pCAR1 : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 466
 4562 Sty : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 512
 1138 Hso : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 512
 106 pWVO : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 516
 3602 Bfu : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 517
 4237 Rme : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 517
 5177 Pfl : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 513
 2259 Xac : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 359
 4044 Psy : --PRTS LMSV NADQVYL RSLAPM GST LSL LADIA TA EINHRLP-----KIMHEDTAA : 516

TraG R478 : --ANNHSMNLLAQ RAK ALFIC LAFI AAS-VET NR T LO YIS NDTP QTI VENFGKSA STNMVYTT : 594
 TraG R27 : --ANNHSMNLLAQ RAK ALFIC LAFI AAN-AET NR T LO YIS NDTP QTI VENFGKSP STNMVYTT : 594
 TraD SXT : --VNDPFCOLL RAK RCVIA FAFSA T SE R LIA MDAE QYV JDN KTR QYIMQTC : 535
 TraD R391 : --VNDPFCOLL RAK RCVIA FAFSA T SE R LIA MDAE QYV JDN KTR QYIMQTC : 542
 5679 Rme : --ANEJLLOLL RAK FKAIVAY RLFTH L NV MQ QIV EDID AQWFAKAGTTA RNLV/S : 562
 TraG pHG1 : --ANEJLLOLL RAK FKAIVAY RLFTH L NV MQ QIV EDID AQWFAKAGTTA RNLV/S : 561
 202 Rts1 : --VNIJVKML SR AFCEMFIA GTAFHA SE ME V THFTA TPAPKAQKA MER PLTKFKYIMRGG : 560
 TraG pCAR1 : --VNIJVKML SR AFCEMFIA GTAFHA SE ME V THFTA TPAPKAQKA MER PLTKFKYIMRGG : 547
 4562 Sty : --AGEEHEMI GAE EVTAY WRIIA I MA A T T MIM REKI AEI IQEPEVE YTKTV : 593
 1138 Hso : --AGEEHEMI GAE QVTAY WRIIA I NR E T T MIM REKI AEI IQEPEVE YTKTV : 593
 106 pWVO : --AGEEHEMI GAE QVTAY WRIIA I NR G T T MIM REKI AEI IQEPEVE YTKTV : 597
 3602 Bfu : --AGEEHEMI GAE QVTAY WRIIA I NR G T T MIM REKI AEI IQEPEVE YTKTV : 598
 4237 Rme : --AGEEHEMI GAE QVTAY WRIIA I NR G T T MIM REKI AEI IQEPEVE YTKTV : 598
 5177 Pfl : --AGEEHEMI GAE QVTAY WRIIA I NR G T T MIM REKI AEI IQEPEVE YTKTV : 594
 2259 Xac : --AGEEHEMI GAE QVTAY WRIIA I NR G T T MIM REKI AEI IQEPEVE YTKTV : 440
 4044 Psy : --AGEEHEMI GAE QVTAY WRIIA I NR G T T MIM REKI AEI IQEPEVE YTKTV : 597

TraG R478 : TGSEIS-LPHNN GSIGE KOTILEESIPKELGGG MFHIVARIQD RKVVGGIPTVAEKMKPNTTLESLFKKACK : 674
 TraG R27 : TGSEIT-LPHNN GSIGE KOTILEESIPKELGGG MFHIVARIQD RKVVGGIPTVAEKMKPNTTLESLFKKACK : 674
 TraD SXT : M SN--DSPAL S HGE MEEEGD FPQIQGG NL YIAKLS R IKG IRLTSSTQAV----- : 699
 TraD R391 : M SN--DSPAL S HGE MEEEGD FPQIQGG NL YIAKLS R IKG IRLTSSTQAV----- : 699
 5679 Rme : T TG-TEAHIGE GSVERSAQLEK/V PHDLAQG NL YIAKLS A VGG IRLTQD----- : 622
 TraG pHG1 : T TG-TEAHIGE GSVERSAQLEK/V PHDLAQG NL YIAKLS A VGG IRLTQD----- : 622
 202 Rts1 : S SG---TDPEQI GSIGE MEEEGD FPQIQGG NL YIAKLS R IKG IRLTSSTQAV----- : 699
 TraG pCAR1 : N AG---EGGVVQGG VGE MEEEGD FPQIQGG NL YIAKLS R IKG IRLTSSTQAV----- : 699
 4562 Sty : V DI INFDQGGD S VQD VSTRVF LS ABQTH NG AFALLE R HKI MPLPASVDCEMVTYLOS VITMRKH : 674
 1138 Hso : F DSINPDQDKA SNTGE ISQKE P DVAMTH NG AFILIN ST YIV MPLPAIDKEDVITASHQELDKMKKH : 674
 106 pWVO : A DSDPHGNTA SNTQD ISSNEV IEIAPVTH NG CFAITE N HKV MPLPAIDKEDVITASHQELDKMKKH : 674
 3602 Bfu : A DSDPHGNTA SNTQD ISSNEV IEIAPVTH NG CFAITE N HKV MPLPAIDKEDVITASHQELDKMKKH : 674
 4237 Rme : A DSDPHGNTA SNTQD ISSNEV IEIAPVTH NG CFAITE N HKV MPLPAIDKEDVITASHQELDKMKKH : 674
 5177 Pfl : A DSDPHGNTA SNTQD ISSNEV IEIAPVTH NG CFAITE N HKV MPLPAIDKEDVITASHQELDKMKKH : 674
 2259 Xac : A DSDPHGNTA SNTQD ISSNEV IEIAPVTH NG CFAITE N HKV MPLPAIDKEDVITASHQELDKMKKH : 674
 4044 Psy : ANDS--ATGNTA SSSHQD VQWVSV IEIAPVTH NG CFAITE N HKV MPLPAIDKEDVITASHQELDKMKKH : 674

TraG R478 : VT-----LRQNLDIKN NKY RKLH----- : 694
 TraG R27 : VT-----LRQNVGLSY NKY RKLH----- : 694
 TraD SXT : ----- : -
 TraD R391 : ----- : -
 5679 Rme : ----- : -
 TraG pHG1 : ----- : -
 202 Rts1 : Y-----EKKVRCEK/RKLANA----- : 655
 TraG pCAR1 : ----- : -
 4562 Sty : Y----RTGDTWKGGSVLPQ-----DNVLDAL----- : 696
 1138 Hso : Y----HVATNWEFAFKDYTPSKD ADSFENMVTEEMKAKADLNWGAIDLPEDDEAESISQADTDEQDMSEF : 743
 106 pWVO : Y----VEAGDWNENQSLPGLQDPA PDDLDDFR-----QMVAA-----EEAEA----- : 718
 3602 Bfu : Y----AEAGDWNENQSLPGLQDPA PDDLDDFR-----HLAAR-----DTAVDGEARF----- : 724
 4237 Rme : Y----SEATQWWEFTSSPALQDGA PDDLDDAAAVEPGLVAT-----DDAGNEASP----- : 728
 5177 Pfl : Y----SEATQWWEFTSSPALQDGA PDDLDDAAAVEPGLVAT-----DDAGNEASP----- : 728
 2259 Xac : Y----VEAGDWNENQSLPGLQDPA PDDLDDAAAVEPGLVAT-----DDAGNEASP----- : 728
 4044 Psy : Y----VEAGDWNENQSLPGLQDPA PDDLDDAAAVEPGLVAT-----DDAGNEASP----- : 728

B.

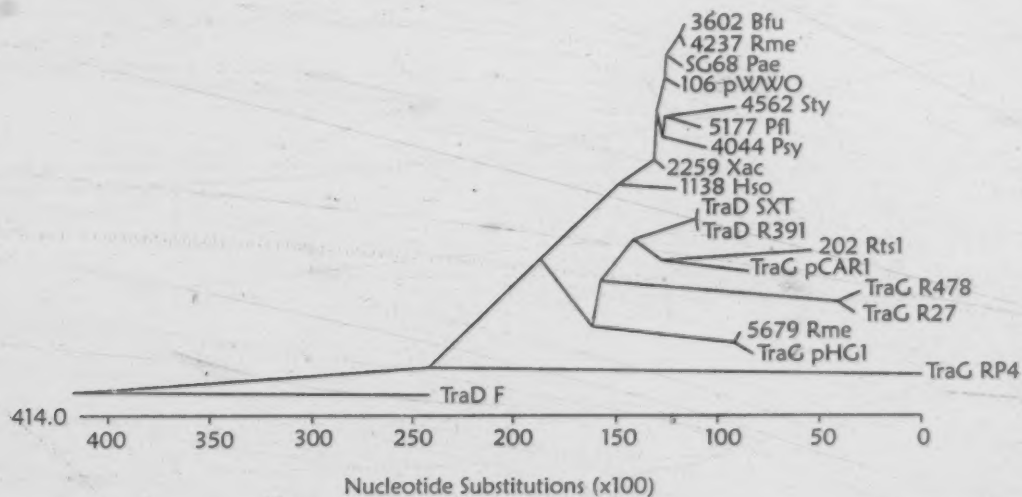


Figure 2-7. Multiple sequence alignment and second structure analysis of TraJ and homologues. Bold/underlined residues indicate transmembrane helices (TMH) predicted by TMHMM. The schematic of TraJ represents predicted TMH (cylinders), cytoplasmic domains (inside), and periplasmic domains (outside). Accession numbers: R478 (CAE51739); R27 (NP_058330); SXT (AAL59721); R391 (AAM08039); pCAR1 (NP_758667); Rts1 (NP_640164); pHG1 (AAP86118); *Haemophilus somnus* (ZP_00123133); *Xanthomonas axonopodis* pv. *citri* (NP_642577); *Pseudomonas fluorescens* (ZP_00087889); *Burkholderia fungorum* (ZP_00030777); *Pseudomonas aeruginosa* (AAN62289); *Ralstonia metallidurans* (ZP_00026655, *note: the *Ralstonia metallidurans* coding sequence 5682 has been re-annotated from the entry at NCBI to include 19 more codons and an alternate start codon).

inside

TMH1

outside

TraJ R478 : AADNTSPAAVRI PFFG IYFISLA----FQMTD---ELKVO TOQ-----NASRS SNDNRKSVIANSEATLNRLVHDYK IDI NTILIP--DTKPPA--RCINIVAEKETS
 TraJ R27 : AADNSRAPIRIG ILLG LY IYSLG---FQNTD---ELKQO TOQ-----NASRS SNDRLSVIANSEATLSTLINDYK LIDY NTILIP--DT--TPA--RCINIVAEKETS
 5043 SXT : SKHKSVMWLLC AIMIE IA GV-----LVPGD---WTR SRK K-----OHQNO GAOTSYWIGTSHRQ QSNV D QUES RDELIPTEOBLRS--KCNENMGGMFV
 36 R391 : FMSKHSVMWLLC AIMIE IA GV-----LVPGD---WTR SRK K-----OHQNO GAOTSYWIGTSHRQ QSNV D QUES RDELIPTEOBLRS--KCNENMGGMFV
 124 PCAR1 : LEEKWPKGVSIAFFV IM AT-----VAPNK---LVDT LRK-----KMGIEI GDSOMEKVLKTNRY SALV D JAKV SDMLHP---RGSTV--DAFENKIDMAVFR
 204 Rts : LQDLKDDKWPISFKGLM WATG MV MF-----FVPAS---FVYON IRE-----EMQOKY GTTEFPDVMVTNAR KYFF D G.DSA XNYKPIKAK'NTG--TGADNG--ASD
 369 PHG1 : ANSBFASHVELML ESPL CT AP-----FIQDQ---SAFE SEAR SRK R-----ASVEGT GTEKADIYAVASANAR RENE D J--A KASFAG-----DAB--TAFSDGG--AST
 5682 Rme* : ASSRFASHVELML ESPL CT AP-----FIQDQ---SAFE SEAR SRK R-----ASVEGT GTEKADIYAVASANAR RENE D J--A KASFAG-----DAB--TAFSDGG--AST
 SG67 Pae : KDPAAATQREOTRQOGLLAGIVTLPRLMLG LIGS LS LIECVGHHLFWDQGRHAQG DYENHLSHHTFSA VOZPGRYAHOLVESA QMLF K CLDM SQASRSRSPHDDARDARYISQVYV
 1136 Hso : PQQTN---QKVKKKGLYITISAL---LATFLS LS LLEWLGIAFIWEQQGHLHSQA IQGMSISETTSRSGYSESELANITLTLHDMLF K G-----FQD---WHSPPKHS--DWEYLI--YH-
 5176 Pfl : VATSQNTPPQPIORPGLIISTISILVRVIG LIAS FS LIEFAGLLFMDQGLHSQA ISLGLWSEHFKSS IQCPGPTIVQMLLOLLHLL K G-----FADFAQOARVSCMG--SFGXINQYV
 2260 Xx : SDVAATQROQVROOGLIGLIVLPERCFG LGS FLC VIECIGHALFMDQGRHAQA GHE DOLSDYFVRS VOI PGRTAHQVIOHATEWAF R G LLEN QDASROARAGASGRPSLPVLTATVSM
 3601 Btu : KDAASTAQROQROGLIVGTITLTPRL LG LIGS FS VVECVGHHLFMDQGRHSQQ QY GHLSSHFTSR VRI PGRTAHQVIOHATEWAF R G LLEN QDASROARAGASGRPSLPVLTATVSM

inside

TMH2

outside

TMH3

inside

TMH4

TraJ R478 : INYTNKNIPL QSPFEN GIVLEPLFAMLA IYCHK VFKV VQYRINFRAN-VISALTNVIV ENSLFNNTAQ FPPVAL GHALNLSNFO L : 220
 TraJ R27 : VNTVAKNPL QSPFEN GIVLEPLFAMLA IYCHK VFKV VQYRINFRAN-VISALTNVIV ENSLFNNTAQ FPPVAL GHALNLSNFO L : 220
 5043 SXT : EVEDRIQAFEDY QVETTFAL VM PFALILMPALM CL TTK TTFDS PIHRYSMII G-SGVILLMGFEA-----IA PFL LPS-LI G ADIAG ALSHQ K : 211
 36 R391 : EVEDRIQAFEDY QVETTFAL VM PFALILMPALM CL TTK TTFDS PIHRYSMII G-SGVILLMGFEA-----IA PFL LPS-LI G ADIAG ALSHQ K : 211
 124 PCAR1 : ILESRGEAVOKI VOM YIVLE VM PFLAVLVPAIAGI RNNA HGPQYS PFLNNAIV S-WGMLIVISL-----LP PFL LPS-LI G ADIAG ALSHQ K : 212
 204 Rts : FTNGAKVENYL KINTYLSL VM PFLIVGVSIFAGI KMS YTFAYS PFTNRSLEIM G-WGIMLSL-----LP PFL LPS-LI G ADIAG ALSHQ K : 212
 369 PHG1 : FGRSRHQHFLT IRA YRANQHYN VGAILVLTALFN SRK AGRCAFPVPSFVAAHG M-LCFPGASL-----LP LATWTFVC GLECKR AASTHVCK : 205
 5682 Rme* : FGRGWQHEFLT IRA YRANQHYN VGAILVLTALFN SRK HAASATFANPVSFVAAHG M-LCFPGASL-----LP LATWTFVC GLECKR AASTHVCK : 186
 SG67 Pae : WSERVLIAAFTLTF VSLV LT PFLIAAFVGHV RRD FGAKRE GFTHRAKAS MFLAVLFWITIT-----TS HFL VILPSAA GHAUN TAASTFK H : 249
 1136 Hso : YRVLIESIIVY ITP VRLM FISEPFLIAAFAGETH RRD FGAKRE GFTHRAKAS MFLAVLFWITIT-----TS HFL VILPSAA GHAUN TAASTFK H : 249
 5176 Pfl : SIEDT'LAAYVITTF VRLM LATP'FLIASLGTFI RRD LGAKRE GFTHRAKAS MFLAVLFWITIT-----TS HFL VILPSAA GHAUN TAASTFK H : 249
 2260 Xx : HAEGYLIAAAT LLF VRLM CLT PIFALAAFGVLV RRD FGAKRE GFTHRAKAS MFLAVLFWITIT-----TS HFL VILPSAA GHAUN TAASTFK H : 249
 3601 Btu : WTESIILAAFTTTF VRLM LT PLISTAAFGVLV RRD FGAKRE GFTHRAKAS MFLAVLFWITIT-----TS HFL VILPSAA GHAUN TAASTFK H : 249

Burkholderia fungorum, *Pseudomonas aeruginosa*, and *Ralstonia metallidurans* (Table 2-2). This indicates that *tral-G-J* sequences have been co-inherited by numerous genetic elements from a common ancestor. When the list of genomes having H-type Dtr/coupling determinants is compared to the genomes having F-like Mpf genes, there is only overlap with the highly related H plasmid, SXT, R391, Rts1, and pCAR1 elements. Otherwise, plasmids pNL1, pRS241d, and the F-like plasmids have F/H-type Mpf genes, but not the H-type *tral-G-J* genes. These observations indicate that the IncH plasmid Mpf and Dtr/coupling determinants have evolved from different lineages.

It was previously reported that relaxosomal and coupling determinants of the IncH plasmids were derived from an IncP lineage (Lawley *et al.*, 2002a). This classification was made partly because the R27-encoded relaxase protein Tral lacks the C-terminal helicase domain encoded on the F plasmid Tral and has the conserved histidine triad motif present in IncP Tral sequences (Figure 2-5) which is involved in Zn^{2+} co-ordination in the active site (Guasch *et al.*, 2003). There is however, little overall sequence identity between Tral_H and Tral_P (14% identity in a global alignment comprised of 46% gaps). In contrast, each member of the H-type Tral family have multiple conserved domains outside of the previously described relaxase motifs, and in a phylogenetic tree, cluster together outside the IncP (RP4) and IncF Tral sequences (Figure 2-5). The coupling proteins of the H-type Dtr/coupling group are also more similar to each other, ranging from 23-31% identity (Figure 2-6, Table A-1), than to TraG of IncP plasmid RP4 (15% identity) or TraD of F (16% identity). There is also conservation of predicted transmembrane and periplasmic domains (Figure 2-6). TraJ is unique to the H-type Dtr/coupling group, and all of the TraJ homologues are of similar length, all have four predicted transmembrane helices, and these helices are in relatively the same site as they are predicted to occur in R478-encoded TraJ (Figure 2-7). In addition, between predicted transmembrane helices number 3 and 4 (Figure 2-7), there is a conserved

Table 2-2. Co-inheritance of *tral*, *traG*, and *traJ* sequences. Homologues to R478-encoded relaxosomal proteins and the coupling protein were detected in the protein database at NCBI. For those genomes described in *genus species* format, these are preliminary annotations deposited to NCBI, and may represent chromosomal or plasmid sequences. Accession numbers: R478 (CAE51736, CAE51737, CAE51739); R27 (NP_058333, NP_058332, NP_058330); SXT (AAL59675, AAL59680, AAL59721); R391 (AAM08003, AAM08004, AAM08039); pCAR1 (NP_758664, NP_758665, NP_758667); Rts1 (NP_640161, NP_640162, NP_640164); pHG1 (AAP86114, AAP86115, AAP86118); *Haemophilus somnus* (ZP_00123099, ZP_00123135, ZP_00123133); *Xanthomonas axonopodis* pv. *citri* (NP_642513, NP_642576, NP_642577); *Pseudomonas fluorescens* (ZP_00087872, ZP_00087890, ZP_00087889); *Burkholderia fungorum* (ZP_00030759, ZP_00030778, ZP_00030777); *Pseudomonas aeruginosa* (AAN62266, AAN62290, AAN62289); *Ralstonia metallidurans* (ZP_00026652, ZP_00026653, ZP_00026655).

Genome	<i>tral</i> ^a	bp between <i>tral</i> - <i>traG</i> ^b	<i>traG</i> ^a	bp between <i>traG</i> - <i>traJ</i> ^b	<i>traJ</i> ^a
R478	<i>tral</i>	0	<i>traG</i>	1095	<i>traJ</i>
R27	<i>tral</i>	10	<i>traG</i>	1089	<i>traJ</i>
SXT	<i>tral</i>	69	<i>traD</i>	557	043
R391	<i>tral</i>	48	<i>traD</i>	547	36
pCAR1	<i>tral</i>	-4	<i>traG</i>	372	124
Rts1	201	-8	202	382	204
pHG1	365	-4	<i>traG</i>	785	369
<i>Haemophilus somnus</i>	1107	27166	1138	334	1136
<i>Xanthom. axonopodis</i> pv. <i>citri</i>	2196	68646	2259	-2	2260
<i>Pseudomonas fluorescens</i>	5158	18591	5177	90	5176
<i>Burkholderia fungorum</i>	3583	16902	3602	-4	3601
<i>Pseudomonas aeruginosa</i>	SG44	18376	SG68	-4	SG67
<i>Ralstonia metallidurans</i>	5678	-2	5679	805	5682 ^c

a. gene designation of homologous sequences are as reported in the accession file listed in the legend

b. overlapping coding sequences are marked with a negative value

c. the annotation of *Ralstonia metallidurans* coding sequence 5682 has been re-annotated from the entry at NCBI to include 19 more codons and an alternate start codon

tandem of positively charged residues (Lys-Arg), which may play a role in orienting the protein in the membrane (i.e. positive-inside rule), or substrate interactions. Helix-turn-helix and leucine zipper motifs were previously predicted for TraJ (Lawley *et al.*, 2002a), but these domains were not detected in the current analysis. The apparent membrane-localization of TraJ is a feature not normally associated with Dtr components, and this essential transfer protein may play a role in the membrane-targeting of the relaxosome during conjugation (J.E. Gunton and D.E. Taylor, unpublished data).

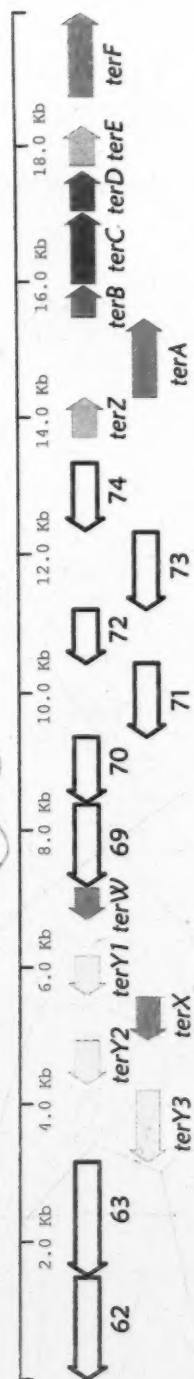
2.3.4 Resistance determinants

Resistance mechanisms for both antibiotics and toxic metals in environmental bacteria pre-date human surveillance, but the combination of selective pressures and horizontal gene transfer have provided the motive and means, respectively, to cause rapid dissemination of these traits throughout the bacterial population, including medically relevant pathogens (Davies, 1994). This is demonstrated by the appearance of multiple resistance phenotypes in *Salmonella* and *Serratia* species due to the carriage of H plasmids. The resistance determinants on R478 are encoded primarily in one contiguous region from co-ordinates 61238-162175. The exceptions are a Tn10 insertion encoding tetracycline resistance and a secondary tellurite-resistance operon that lie outside of this region (Figure 2-1).

2.3.4.1 Tellurite, bacteriophage, and colicin resistance

Tellurium (Te) compounds have a long standing history as therapeutic agents and as selective agents for the isolation of pathogens (Taylor, 1999). Five distinct groups of plasmid and chromosomally-encoded tellurite resistance determinants have been identified (Taylor, 1999). The IncHI2-type determinants are characterized by a conserved set of sequences (*terZABCDEF*) found on numerous IncHI2 plasmids isolated from different bacterial hosts (Hou and Taylor, 1994) (Figure 2-8). These determinants

Figure 2-8. Genetic organization of the operons encoding tellurite resistance, Phi and Pac. Individual coding sequences are represented as arrows, and coloured arrows have been previously characterized, except for TerY2 and TerY3, which are similar to TerY1. Genes are transcribed in the direction indicated by the respective arrow.



provide a high level of resistance to tellurite oxyanions (MIC ~1024 µg/ml) by lessening the oxidation of thiol groups (Turner *et al.*, 1999), however the mechanism of this protection is unknown.

Besides providing resistance to tellurite, the IncHI2-type determinants inhibit host cell infection by bacteriophage Lambda, T1, T5, T7 and φ80 (Phi phenotype), and resistance to pore-forming colicins A, B and K (PacB phenotype) (Taylor and Grant, 1976; Whelan *et al.*, 1995). TerC, TerD and TerZ appear to be the principle determinants, since transposon mutants of the respective genes were reduced in or lacked each of the above phenotypes (Whelan *et al.*, 1995). It was also noted that cloning of *terZABCDE* in a multicopy vector resulted in a lethal phenotype and filamentous morphology of the host cell; and the inclusion of an additional DNA segment containing sequences for *terWYX* 'protected' cells from these effects (Whelan *et al.*, 1997). TerW contains a predicted helix-turn-helix and is a member of COG2378 (transcriptional regulator), corresponding to the previous prediction that TerW mediates protection from TerZABCDE by modulating gene expression (Whelan *et al.*, 1997).

Tellurite resistance determinants related to the IncHI2 determinants are present in a large number of chromosomal sequences, including many strains of *E. coli* O157:H7, *Yersinia pestis* and the genus *Proteus* (Taylor *et al.*, 2002; Toptchieva *et al.*, 2003) (Table 2-1). *E. coli* and *Y. pestis* sequences include both the *terW* and *terZ* divergently opposed operon sequences, and the six genes of unknown function present in the interceding region between *terW* and *terZ* (Figure 2-8), implying that that this entire region has transferred between numerous bacteria and genetic elements. Using the complete sequence of R478, there may be additional genes involved in mediating the phenotypes of tellurite resistance, Phi, and PacB. Two additional copies of *terY* were detected (and the *terY* genes have been renamed *terY1*, *terY2*, and *terY3*), and at the

end of this putative operon (Figure 2-8) there are two coding sequences (SMR0062/63) with strong sequence identity to known serine/threonine kinases and phosphatases. Amino acids 140-273 of SMR0062 align with the protein kinase domain pfam00069 (e value: $2e-8$), and 298-545 of SMR0063 align with the phosphatases domain cd00143 (e value: $6e-4$). This may represent a signaling module responsive to a yet unknown signal.

A surprise on the R478 sequence was the detection of IncP-type tellurite determinants *klaABC* (otherwise called *kilA*, *telA* and *telB*). The R478 coding sequences SMR0256-258 are 23-57% identical to full-length coding sequences encoded on the pseudomonad plasmids pCAR1 (Maeda *et al.*, 2003) and RK2, and it was previously found that all three RK2-encoded determinants are essential for resistance to tellurite (Goncharoff *et al.*, 1991; Maeda *et al.*, 2003; Turner *et al.*, 1994). The two essential cysteine residues present in TelB (Turner *et al.*, 1994) are lacking in both the pCAR1 and R478 *KlaC* sequences, and *klaC* is the least conserved gene of this tripartite operon, indicating that if functional, this module may have a different mode of action. No obvious insertion elements surround this cassette of resistance genes, nor do any other genes that might point towards a Pseudomonad origin (Table A-1). Additionally, the *klaABC* genes are not present in either R27 or pHCM1.

2.3.4.2 Mercury resistance

Toxicity to inorganic mercuric ions results from Hg(II) binding with high affinity to protein thiol groups (Silver and Phung, 1996). The *merTPCAD* and *merR* genes encoded by R478 (SMR0118-223) are highly similar in both organization and sequence to respective sequences on R100 (Tn21), R391, pDU1358, pSB102, Tn501, pKLH201, pKLH256, and other genetic elements (Table 2-1). Mercury resistance is mediated by efflux, and the structural genes for Hg transport being *merTPC*, with the latter gene

being absent from some *mer* resistance operons. After transport from the cytosol, MerA, the mercuric reductase volatilizes Hg^{2+} to the less reactive Hg^0 gas. Both *merD* and *merR* are regulatory genes (Summers, 1992) that are not part of the *mer* operon. The most striking change in G+C content on R478 occurs within the *mer* region, the left border of which has been disrupted by transposable elements which are now themselves present as pseudogenes (Figure 2-1).

2.3.4.3 Silver resistance

Silver compounds are used in hospitals, industry, and agriculture as antimicrobial agents due to their bactericidal effects (Gupta and Silver, 1998). The initial identification of a silver resistance operon (*sil*) encoded on an IncHI2 plasmid was after isolation of pMG101 from a nosocomial outbreak strain of *S. enterica* sv. Typhimurium (Gupta *et al.*, 2001). In this study, *sil* sequences were also detected on R478, as well as resistance to silver cations by cells harbouring R478. Silver resistance was found to be inducible by exposure to silver cations, possibly due to the presence of a two-component regulatory system comprised of an inner membrane-associated sensor histidine kinase (SilS) which phosphorylates and activates the respective cytoplasmic response regulator (SilR), which in turn activates transcription of the structural genes (*silABC*). The SilABC proteins form a tripartite, membrane-spanning chemiosmotic cation/proton antiporter (SilA: inner membrane antiporter; SilB: periplasmic-spanning protein; and SilC: outer membrane protein). Also present in the pMG101 sequence is a P-type ATPase (SilP) related to other cation transport ATPases, therefore multiple mechanisms for silver cation export seem to be present. The sequence data for R478 confirms that all of the *sil* sequences present on pMG101 are highly conserved on R478 (SMR0141-149 range from 92-100% amino acid identity compared to pMG101 Sil sequences). These two

InchI2 plasmids are likely to be very closely related, as pMG101 similarly encodes resistance to mercury, tellurite, chloramphenicol and tetracycline (McHugh *et al.*, 1975).

Encoded upstream of the R478 *sil* sequences are the Tn7 transposition genes *tnsABCD* (SMR0132-135). A complement of TnsABC+E are generally observed for Tn7 insertions into conjugative plasmids, whereas the TnsABC+D array is involved in recognition of *attTn7* for insertion into bacterial chromosomes (Peters and Craig, 2001). The R478-encoded sequences are also atypical in that there is a low level of sequence conservation (ranging from 21-39% overall length-wise identity), and neither the tandem 22-bp repeats at the Tn7 left and right border, or an *attTn7* site (Peters and Craig, 2001) were observed. However, TnsC has retained the characteristic Walker A NTP-binding domain, and TnsD has a helix-turn-helix appropriate for a DNA-binding protein (data not shown).

2.3.4.4 Copper resistance

Although transition metals such as copper and zinc are essential for function of many oxidizing enzymes, in excess they are toxic to cells due to non-covalent interaction with cysteine and histidine residues (Gatti *et al.*, 2000). The mechanism of copper tolerance was determined to be copper sequestration in the periplasm by CopA and CopC (Cha and Cooksey, 1991). A two-component regulatory system, CopS and CopR, controls expression of the structural genes (Munson *et al.*, 2000). The putative copper tolerance determinants encoded on R478 (CopABCDRSE, SMR0153-159) are 92-100% identical to the PcoABCDRSE proteins encoded on pRJ1004 (Brown *et al.*, 1995), a 125 kbp conjugative plasmid belonging to incompatibility groups I1 or K (Tetaz and Luke, 1983). Furthermore, the two-component regulatory system of the *cop* operon is similar to that regulating silver resistance operons (see above), the *E. coli* chromosomally-encoded YlcA and YbcZ (Munson *et al.*, 2000), and the CzcRS proteins which promote

expression of *czcABC* encoding a cation-proton antiporter in *Ralstonia metallidurans* (Nies, 1995). These latter functions are encoded on pMOL30, a 240 kbp self-transmissible plasmid that also encodes resistance to mercury, zinc, cadmium, and cobalt (Mergeay *et al.*, 1985). Plasmid-encoded copper resistance mechanisms are apparently highly conserved, and are controlled by a two-component system utilized by numerous resistance operons.

2.3.4.5 Arsenic resistance

Ryan and Collieran (2002) characterized the R478 resistance determinants for arsenate and arsenite by using a 5.5 kbp subclone containing six R478 coding sequences (*sfpAB* and *arsCBRH*, SMR0174-179), for which they could detect similar sequences on numerous other IncHI2 plasmids. Interestingly, arsenic resistance is mediated by *ArsC* which reduces arsenate, As(V), into the more toxic arsenite, As(III), which is then transported out of the cytosol by *ArsB*. R478, like the Staphylococcal plasmids pSX267 and pl258 (Silver and Phung, 1996), does not encode the P-type ATPase *ArsA* which interacts with *ArsB* for anion transport. Consequently, arsenite must be transported chemiosmotically. *ArsH* has an unknown function, but is required for arsenic resistance in *Yersinia* carrying plasmid pYV (Neyt *et al.*, 1997). *ArsR* acts as a transcriptional activator for the *ars* operon, and possibly for the divergently transcribed *arsH* (Ryan and Collieran, 2002). Furthermore, *ArsR* is thought to co-regulate *sfp* and *ars* operons (both encoding anion transport systems), as binding sequences of this regulator are also found upstream of the *sfpA* gene (Ryan and Collieran, 2002). Immediately after the R478 *arsH* sequence is a transposase sequence (similar to the IS1202 transposase), and this region also has an elevated G+C content. Each of these characteristics suggest that this resistance module was acquired as an

insertion, possibly from a sequence related to Tn2502 on plasmid pYV (Neyt *et al.*, 1997).

2.3.4.6 Tetracycline resistance

The common resistance determinant that is present in all three sequenced IncHI plasmids is Tn10, encoding *tetRACD* (R478: SMR0220-223). These genes encode a tetracycline efflux system, in which TetA is the integral membrane transporter, TetR represses expression of the operon, and TetC and TetD have unknown function (Lawley *et al.*, 2000). The *tet* genes are bounded on each side by insertion sequence elements (IS10L and IS10R), which cumulatively represent a composite transposon. Tn10 is also present on plasmid R100 (accession: NC_002134). Despite being common to all H plasmids, Tn10 is present in a different location in each plasmid, implying that Tn10 was not present in an ancestral H plasmid, and has been independently acquired by each of these plasmids. Additionally, a subsequent insertion in *tetC* of pHCM1 by an IS1-related element disrupted *tetC* and lead to the deletion of *tetD*. The IS10R sequence is still present in pHCM1, and the intervening sequences between IS10R and the *tetC* pseudogene include the numerous resistance determinants encoding β -lactamase, chloramphenicol acetyltransferase, streptomycin phosphotransferase and a mercury resistance operon, which account for the majority of the differences between pHCM1 and R27.

2.3.4.7 Chloramphenicol and kanamycin resistance

Similar to the insertion sequence-rich region of pHCM1, R478 encodes resistance determinants *cat* (chloramphenicol acetyltransferase, SMR0108) and *aphA* (an aminoglycoside phosphotransferase that provides resistance to kanamycin, SMR0110). In R478 these sequences are surrounded by five identical IS26 elements (Figure 2-9). The IS26 sequences appear to have disrupted the existing IntI1 class 1 integrase

Figure 2-9. Genetic organization of R478 sequences encoding *cat*, *aphA*, and IS26.

Individual coding sequences are represented as arrows, and pseudogenes are coloured brown. Genes are transcribed in the direction indicated by the respective arrow.

(SMR0096) and recombinase/resolvase (SMR0096b) elements, revealing a succession of insertion events in this region as part of the expansion and gene loss that has occurred during the evolution of R478.

The determinants for metal and antibiotic resistance encoded on R478 may have been acquired from various other plasmids, as indicated by the presence of orthologous sequences on both related and unrelated plasmids (Table 2-1). It is also apparent that various transposons and insertion elements (Tn7, Tn10, IS26, and IS1202) have contributed to the acquisition of these determinants on R478. Consistent with this notion is the observation that each of the resistance determinants have markedly higher G+C content than the R478 average.

2.3.5 Other functions

Numerous proteins with putative DNA-binding motifs are encoded by R478 (Table 2-1). It is possible that these genes have been steadily acquired and maintained because of potential roles in regulation (i.e. H-NS, TlpA, and Hha), stability and modification (i.e. several putative nucleases and DNA methylases) of this large extrachromosomal genetic element, or that they may be involved in plasmid replication (i.e. presence of a putative helicase and a primase, see Table 2-1). There are also several genes predicted to encode enzymes which may be required for the function of other plasmid-encoded proteins (i.e. putative disulphide isomerases, Table 2-1).

Upstream of the Tra1 region are a conserved set of coding sequences (SMR0191-193) that are similar to a putative retroelement (coding *retA* and *mucAB*) encoded in the IncL/M plasmid R471a, isolated from *S. marcescens*. The *retA* gene encodes a putative reverse transcriptase (RetA) and *mucAB* encode homologues of the chromosomally-encoded UmuCD mutagenesis proteins (Kulaeva *et al.*, 1998). In plasmid R471a, *mucB* is followed by a gene encoding the transposase of an IS1396 element, which led

Kulaeva and colleagues (Kulaeva *et al.*, 1998) to suggest that this putative retroelement has been mobilized from a different genome, as *mucAB* are commonly found on plasmids (Table 2-1). Such an insertion sequence is absent in R478, indicating that if these determinants were previously acquired by transposition from R471a, or a similar element through this proposed mechanism, subsequent genomic insertions or deletions in R478 have immobilized the retroelement. Notably, the *mucAB* genes are present in the same location of R27 and pHCM1, although the upstream *retA* gene is lacking. Again, it is possible that subsequent genomic rearrangements have caused the deletion of this feature.

2.3.6 Comparison of three H plasmid sequences

Comparative genomics of microbial chromosomal sequences has permitted the identification of niche-specific, virulence and strain-specific determinants (Deng *et al.*, 2003; Parkhill *et al.*, 2003; Perna *et al.*, 2001). The comparison of related plasmid sequences allows, on a smaller scale, an analysis of evolutionary events which have created transmissible genetic elements encoding multiple resistance phenotypes.

Global sequence comparisons demonstrate that the IncHI2 plasmid R478 is extremely similar to the previously sequenced IncHI1 plasmids R27 (Sherburne *et al.*, 2000) and pHCM1 (Parkhill *et al.*, 2001) (Figures 2-10 and 2-11). The original classification of three subgroups for IncHI plasmids was partly based upon the results of a DNA-DNA hybridization study that suggested IncHI1 and IncHI2 plasmids are unrelated at the DNA sequence level (Roussel and Chabbert, 1978). Another study attempting to determine the relatedness of plasmids in the same incompatibility group found that of the Inc F, I, N, B, and H groups, only members of the IncH group do not share sequence similarity (Grindley *et al.*, 1973). A more recent study concerning a different set of IncHI plasmids also found a low level of similarity between IncHI1 and

Figure 2-10. Venn diagram detailing the unique and shared gene complements of plasmids R478, R27 and pHCM1. Numbers in parentheses indicate the number of insertion sequence elements that are included in the grouping.

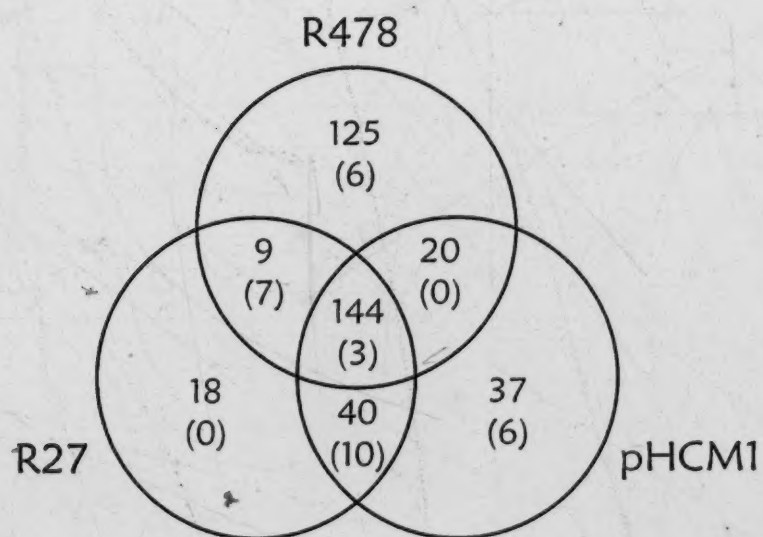
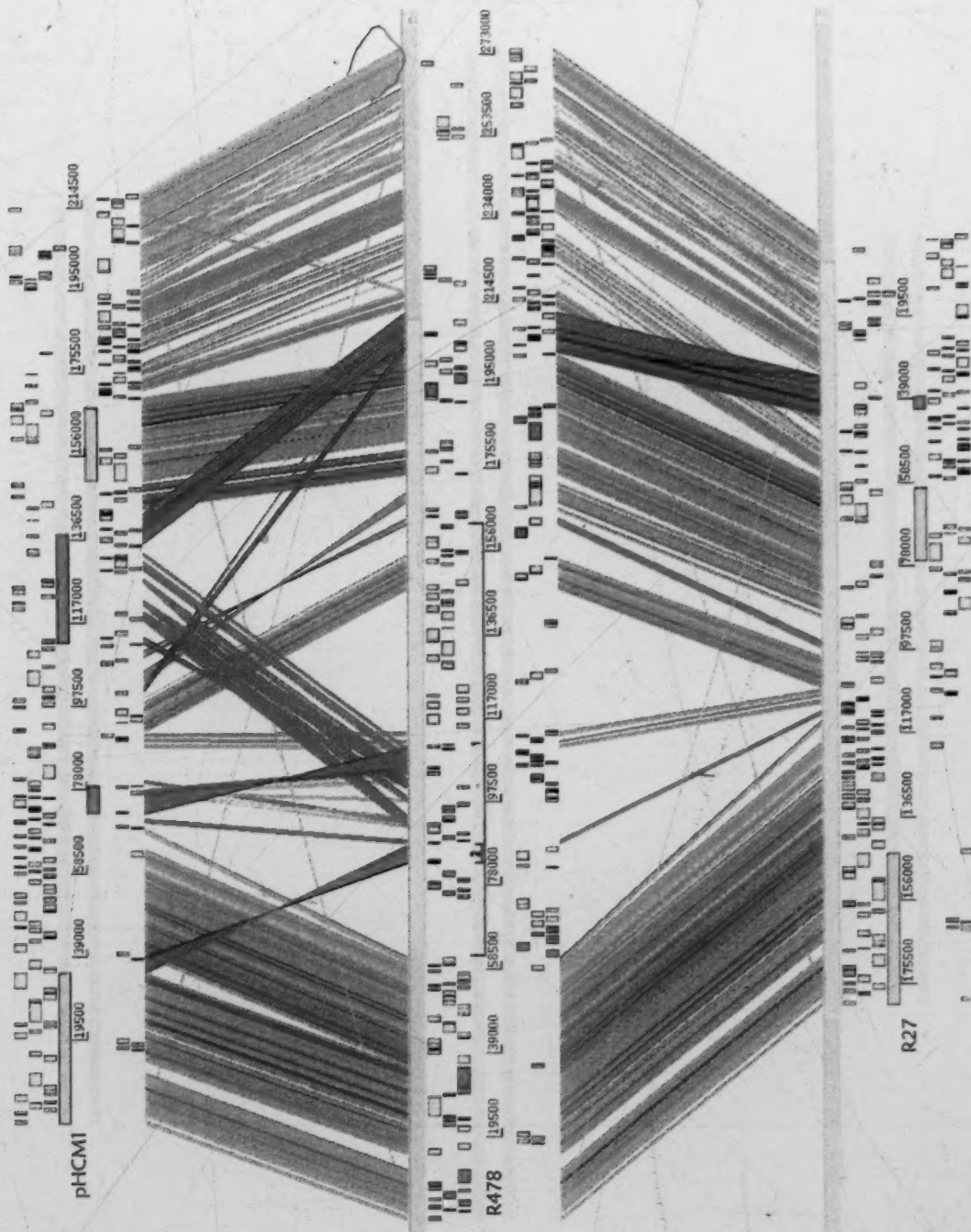


Figure 2-11. Linear genomic comparison of R478 to R27 and pHCM1. The six-frame translation of the R478 sequence was compared to the six-frame translation of R27 or pHCM1 using tBlastX and displayed using the Artemis Comparison Tool (ACT). Regions having similarity above a threshold of approximately 27% identity are connected via a red line. R478 coding sequences are color-coded as follows: red, conjugative transfer; orange, replication; yellow partitioning; purple, tellurite resistance; light pink, insertion elements; dark pink, chloramphenicol resistance; brown, kanamycin resistance; dark green, mercuric ion resistance; grey, silver resistance; light green, copper resistance; blue, arsenic resistance; black, tetracycline resistance; light blue, miscellaneous or unknown function. R27 and pHCM1 core genes (replication, transfer and partitioning) are marked with orange boxes, while resistance genes marked with purple boxes. R478 sub-regions labeled 1 or 2 are discussed in the text.



IncHI2 groups, where R478 restriction digests were probed with radiolabelled IncHI1 plasmid DNA (Whiteley and Taylor, 1983). IncHI1 probes that bound to IncHI2 restriction fragments were attributed to common resistance and transfer determinants (Whiteley and Taylor, 1983). It is likely that DNA hybridization would only be positive with highly related sequences. With the complete sequence data now available, it is clear that there is in fact considerable 'relatedness' between members of different H incompatibility subgroups. This was likely not detected by the less precise methods used in earlier studies.

Approximately half of the R478-encoded genes are also present in both pHCM1 and R27 (Figure 2-10). In particular, there is conservation of both gene order and individual coding sequences in a large contiguous section comprising RepHIA (SMR0001), the *tus* gene, partitioning modules Par1 and Par2, the Tra2 conjugative transfer region, and the downstream genes (of mostly unknown function) up to and including SMR0061 (Figure 2-11). Another segment (SMR0185-218), comprising the Tra1 transfer region, *mucAB*, and surrounding genes of unknown function, is also highly conserved. It is likely that the common ancestor of R478, pHCM1 and R27 had a genetic organization similar to these conserved regions.

Divergence from this ancestral sequence may have occurred after transfer events, resulting in separate lineages. The principal difference between R478 and the other sequenced H plasmids is a large segment downstream of Tra2 and upstream of Tra1 in which there are few shared sequences. This region is termed the principal plasticity zone, and is labeled as section 1 in Figure 2-11. In this region are encoded the majority of the resistance determinants (*ter*, *cat*, *aphA*, *mer*, *sil*, *cop*, *ars*), and also Tn7 and IS26 sequences. In pHCM1, this same region has been subject to insertion of *bla*, *cat*, *str*, and *mer* resistance determinants (Parkhill *et al.*, 2001), and it also accounts for the primary difference between pHCM1 and R27. Additionally, the ancestor common to

R478, R27, and pHCM1 encoded no resistance determinants, and insertion of each determinant constituted an independent acquisition. Tn10 is the only resistance determinant common to all three sequenced H plasmids, and this transposon is present at unique locations in each plasmid. Notably, R478 coding sequences SMR0088 (encoding a putative ATP-dependent DNA helicase) and SMR0089 (unknown function, but containing a predicted signal peptide cleavage site), which are 99% identical to pHCM1 coding sequences HCM1.102 and 103, also appear to be independent insertions into the principal plasticity zone of each respective plasmid, since they are present in a different genetic context and orientation (Figure 2-11, section 2).

In terms of incompatibility classifications, the absence of both RepHIB and RepFIB replicons in R478, and the presence of the unique RepHI2 replicon, verifies the validity of the HI2 subgrouping, rather than being subsumed into HI1, despite the observed high level of sequence conservation. The RepHI2 replicon (SMR0292) is in a region containing many repeated coding sequences of unknown function (SMR0236, 299, and 304 are similar to each other, as are 280, 290, 293; data not shown) that each have related sequences in R27 and pHCM1 (Table A-1). There are no obvious insertion sequences surrounding RepHI2, but the large number of duplication events in the surrounding region indicate a localized history of genetic rearrangements. Another distinctive feature of this region suggesting prior rearrangements is the absence of insertion sequences adjacent to each side of the putative Hok/sok systems encoded in R27 and pHCM1, whereas SMR0243a/hok is bordered by such features.

The presence of multiple functional replicons may permit the IncHI plasmids to extend both host range and limit incompatibility with other plasmids (Gabant *et al.*, 1993). The evolutionary events resulting in the presence of multiple replicons, however, are unclear. It is plausible that a singular ancestral IncHI plasmid encoded each of the RepHIA, RepHIB and RepHI2 replicons, with subsequent recombination events resulting

in the loss of individual replicons and the generation of the currently observed IncHI plasmids. Alternatively, individual IncHI plasmid species encoding a single replicon may have independently recombined with co-resident plasmids, thereby expanding the number of replicons. Each of the HI replicons are similar to numerous other plasmid replicons (Table 2), and these plasmids (or their ancestors) may have been the source of the present IncHI replicons. This latter possibility is also more likely because no H plasmid carrying all of these replicons has been observed, and the genetic organization around the RepHI2 replicon indicated prior re-arrangements.

Using the sequence data from R478, R27, and pHCM1, and experimental data concerning individual coding sequences, it is possible to define the backbone components for IncHI replication, partitioning and maintenance, and conjugative transfer. The minimal IncHI determinants, as defined by these three H plasmid sequences, include the RepHIA replicon, the essential *trh/tral/oriT* sequences encoded in Tra1 and Tra2, and the Par1 partitioning module. The RepHIA replicons from both R27 and R478 were found to independently support plasmid replication (Newnham and Taylor, 1994; Page *et al.*, 2001), and this was the only common replicon amongst the three sequenced IncHI plasmids. Essential R27 and R478 transfer genes have been determined (Lawley *et al.*, 2002a; Lawley *et al.*, 2003a; Page *et al.*, 1999), and a difference in the level of conservation between essential and non-essential transfer sequences was observed. The higher sequence identity between essential R478 and R27 transfer orthologues implies that these genes are subject to additional selective forces to conserve functionality, impeding the divergence from the ancestral sequence. Similarly, the Par1 regions of R478 and R27 are more conserved than the putative Par2 regions, and the Par1 region of R27 was found to be the principal partitioning determinant (Lawley and Taylor, 2003). The backbone includes no resistance determinants, but rather these are accessory functions which in part distinguish the different plasmid species.

A successful combination of backbone determinants that ensures continuing plasmid survival (through vertical and horizontal transmission) would be expected to be highly conserved. The presence of the Par module in the Tra2 region may ensure the co-inheritance of partitioning and conjugative transfer genes. Similarly, the RepHIA determinant is present immediately upstream of the Tra2 region in all sequenced IncHI plasmids, which may also serve as evidence of co-inheritance. Notably, the putative replication initiator protein RepC of *R. sphaeroides* plasmid pRS241d (accession: ZP_00008306) is also encoded immediately upstream of the H/F-type transfer determinants, and the RepC protein of *A. tumefaciens* plasmid pTiC58 is encoded adjacent to the *trb* transfer determinants (Li and Farrand, 2000). Thomas (2000) has previously suggested that clustering and intricate control networks between the core plasmid determinants promote co-inheritance.

2.3.7 Conclusions

The evolution of R478, R27 and pHCM1 was seemingly predominated by a series of acquisition events, rather than deletion events, as evidenced by the conserved co-linear nature of shared sequences. These shared co-linear sequences include each of the designated backbone determinants. Once a productive backbone of core plasmid determinants has evolved, as demonstrated with the H plasmid backbone, it is likely to be highly conserved because each backbone determinant encodes a function essential for vertical or horizontal transmission. Insertions of accessory determinants into vertical transfer determinants may result in the loss of the individual recipient plasmid from the gene pool, whereas insertions into horizontal transfer determinants may restrict the plasmid to the present host. In contrast, insertion or deletion of accessory determinants in non-core regions may allow conversion to a genetic element conferring novel phenotypic traits. This evolutionary pattern may have resulted in the apparent clusters

of backbone and accessory determinants observed in the H plasmid sequences. R478 may have originated from an ancestral IncHI plasmid, encoding the backbone sequences described above, but independently acquired accessory determinants (including each of the resistance determinants). Likewise, the IncHI1 plasmids R27 and pHCM1 are also derivatives of this ancestral sequence and have also independently acquired accessory determinants. Incompatibility groups are therefore an abstract classification scheme defined by a conserved set of plasmid backbone features, whereas individual plasmid species are, in addition to the backbone, defined by accessory features. The predominance of resistance determinants in IncHI plasmids highlights the influence of the host on the processes of selection and maintenance of plasmid accessory features.

Chapter 3

Visualization and Characterization of Conjugative Transfer

Protein Complex Assembly via a Mpf Protein-GFP Fusion

Portions of this chapter have previously been published as:

Gilmour, M.W., Lawley, T.D., Rooker, M.M., Newnham, P.J., and Taylor, D.E. (2001) Cellular location and temperature-dependent assembly of IncHI1 plasmid R27-encoded TrhC-associated conjugative transfer protein complexes. *Mol Microbiol* **42**: 705-715.

Gilmour, M.W and Taylor, D.E. (2004) A subassembly of R27-encoded transfer proteins is dependent on TrhC nucleotide triphosphate-binding motifs for function but not formation. *J Bacteriol* **186**: In Press.

3. Visualization and characterization of conjugative transfer protein complex assembly via an Mpf protein-GFP fusion

3.1 Introduction

The 238 amino acid (27kDa) green fluorescent protein (GFP) of *Aequorea victoria* has proven useful as a reporter for protein localization in bacteria. Not only does GFP permit *in situ* visualization of fusion proteins in real time, but visualization occurs in a non-invasive fashion in live bacteria without substrate addition (Margolin, 2000). GFP mutants created by Cormack and colleagues (Cormack *et al.*, 1996) provide considerable improvements over wild-type GFP. GFPmut2 (Ser65Ala and Val68Leu) is a red-shifted variant with an excitation maxima at 488nm. At this wavelength GFPmut2 fluoresces 19-fold more intensely than wild-type GFP. This variant protein is also significantly more soluble than the wild-type and has a decreased maturation time, as wild-type GFP is not detectable until 1-2 hours after synthesis, while GFPmut2 fluoresces within 8 minutes (Cormack *et al.*, 1996). These properties permit more accurate time course and localization studies to be conducted than with wild-type GFP.

The proteins which facilitate conjugative transfer of plasmid DNA represent a dynamic module that is functional in the cytosol, cell envelope, and extracellular environment. Besides the pilus, no distinguishable superstructures have been visualized for the conjugative apparatus, although several macromolecular complexes have been proposed to exist. These include the cytoplasmic relaxosome responsible for DNA processing prior to and during transfer (Lanka and Wilkins, 1995), the membrane-associated mating pair formation complex (Mpf) responsible for construction of the pilus and DNA transfer (Grahm *et al.*, 2000), and a multimeric coupling protein which links the relaxosome to the Mpf complex (Gilmour *et al.*, 2003; Gomis-Ruth *et al.*, 2001; Schroder *et al.*, 2002). Despite the absence of a readily visible transmembrane apparatus during electron microscopy, such as that visualized in type III secretion systems (Blocker *et al.*,

2001), several studies have identified specific interactions between type IV secretion proteins or identified functional subgroups indicative of protein assemblies (Gilmour *et al.*, 2003; Grahn *et al.*, 2000; Harris *et al.*, 2001; Liu and Binns, 2003; Ward *et al.*, 2002).

This study focused on the VirB4-related protein TrhC, an inner membrane-associated Mpf protein required for the conjugative transfer of the large IncHI1 resistance plasmid R27 (Lawley *et al.*, 2003a; Taylor *et al.*, 1999). TrhC is encoded in both R478 and R27 (84% identity), and despite being a member of the VirB4 family, is more similar to F-type TraC proteins (Figure 3-1). A TrhC-GFP fusion construct was created which permitted visualization of the cellular location of TrhC-associated protein complexes. TrhC was present in membrane-associated protein complexes, visualized as distinct fluorescent foci at the cell periphery. In addition, the R27-encoded proteins that are required for the formation of TrhC-associated protein complexes were identified, and the role of the Walker NTP-binding motifs within TrhC for protein complex assembly, DNA transfer, and pilus formation were addressed. A key feature of this study is the insertion of *gfp* into R27 plasmid DNA to create a *trhC-gfp* fusion under control of natural expression determinants. This revealed the temperature-dependent expression profile of *trhC* and mechanisms for the thermosensitive transfer of R27.

3.2 Experimental Procedures

3.2.1 Bacterial strains and plasmids

All strains and plasmids used in this chapter are listed in Table 3-1. Antibiotics were used at the following concentrations, when appropriate: ampicillin (Ap) 100 µg/ml, chloramphenicol (Cm) 16 µg/ml, rifampicin (Rif) 20 µg/ml, nalidixic acid (Nal) 30 µg/ml, tetracycline (Tc) 10 µg/ml, and kanamycin (Km) 10 µg/ml. Conjugal matings were performed for the indicated time and carried out as previously described (Taylor and Levine, 1980).

Figure 3-1. Multiple sequence alignment of TrhC-related sequences. The alignment was generated using ClustalW in MegAlign (DNASar) and conserved residues were shaded using GENEDOC. Walker NTP-binding motifs are indicated. Accession numbers: R478 (CAE51543), R27 (NP_058238), SXT (AAL59681), R391 (AAM08001), pCAR1 (NP_758682), Rts1 (NP_640173), *Rhodobacter sphaeroides* (Rsp: ZP_00008315), F (AAC44180), pSLT (NP_490573), R100 (NP_052960), pNL1 (NP_049162).

TrnC R478 : -----MCALNNYNSG NRHOLGGF VYDOLASTHYLIDGNR SFICMRRVDFMQQ : 56
 TrnC R27 : -----MRGANVYKE NRHOLGGF VYDOLASTHYLIDGNR SFICMRRVDFMQQ : 56
 TrnC SXT : -----MKASLYGQRAEL VLA SDDEC FMEDCG SFCLCD FGDSEVA : 51
 TrnC R391 : -----MKASLYGQRAEL VLA SDDEC FMEDCG SFCLCD FGDSEVA : 51
 TrnC pCAR1 : -----MLKALIRTPRLK NGLA EPNLY FOLEGNR AFLECSFSGHSGEG : 52
 213 Rts1 : -----MSESLIVKRLGKIRF DVC VNA DEKNR FLENNY AFDFVCR AUSTGKET : 56
 4264 pRS241d : -----MGVAMSAKALFQGLDFDDESEDEPQAIATDIVAD YRL DPESE FNO-N-T FLEVN VMAOVAS : 73
 TrnC F : -----MNNPLEAVTOAVNSLVLTALKLPDESAKANEVLGEI FPOFARL YRD NOESC FMD-T FLELAIR NAEESIV : 79
 TrnC pSLT : -----MKNLLDTVTOAANSLTALKLPDESAQANQILGE FPOFARL YRD NOESC FMD-T FLELAIR NAEETIA : 79
 TrnC R100 : -----MSNNPLEAVTOAVNSLVLTALKLPDESAKANEVLGEI FPOFARL YRD NOESC FMD-T FLELAIR NAEESIV : 80
 TrnC pNLI : -----MLFGESAHPEAEKPVIGAFMLNW YRS DKKHG FINT-LP FLELAIR NAEESIV : 80

TrnC R478 : DV AE FKMDITDTVCQIS TALP HTLO SANSVAVGGKMTG-----NDKIFADLLNGYQL YDR-SMHR KF : 127
 TrnC R27 : DV TE FKMDITDTVCQIS TALP HTLO SANSVAVGGKMTG-----NDKIFADLLNGYQL YDR-SMHR KF : 127
 TrnC SXT : DF NV LNNDKMDTL FGF YA DQDTE QRMGLL--HQG-----DPLIFASIRK--AD LDG-GIVC EE : 118
 TrnC R391 : DF NV LNNDKMDTL FGF YA DQDTE QRMGLL--HQG-----DPLIFASIRK--AD LDG-GIVC EE : 118
 TrnC pCAR1 : DF KACITVDKREKTI QFS IC ENEMKGTGLRLRAFRSRTDLPVETOALLEKVINAA--AD FSD-FWMP DS : 130
 213 Rts1 : DO KT LSSNRPKTI QWD IA ISNVKK NQMDPIR-INCED-----PILNAYIN--SK LLH-SDYC KG : 123
 4264 pRS241d : HLOAV NSN-AENGAT FJLWNT FHDGQ VRAWRH--LRG-----DEL MEMAQR--MAHLL--GARFGTDH : 137
 TrnC F : EA DH LRTKLRGIP CIH MS QMGDR EYGLRFEF--WSG-----FOAERFNAT--AY KKAATQF PE : 147
 TrnC pSLT : LS DH LRTKLRGIP CVH MS QMGDR EYGLRFEF--WSG-----FOAERFNAT--AY KKAATQF PE : 147
 TrnC R100 : EA DH LRTKLRGIP CIH MS QMGDR EYGLRFEF--WSG-----FOAERFNAT--AY KKAATQF PE : 149
 TrnC pNLI : EL TQSLSDAMSGCE FLIHQ PSGER ADW MFI--VVA-----KCVYGAAMH--AR LR-AAWMS SR : 126

TrnC R478 : DH-DNLM DQVW LSI QS-ALPYELHSPIDS YSELVSK ITIGLHFFKDAEN YC DKVHPGKSRMAEG- : 205
 TrnC R27 : DH-DKLM DQVW FSI QS-ALPSEIEKTRIDA YSELVSK ITIGLHFFKDAEN YC DKVHPGKSRMAEG- : 205
 TrnC SXT : S--TQTC NPQCI CKL EN-PIPIERLSRASA PASFSQA ATVGFRVTE TDRN SA SAQWNGKASRNPS : 196
 TrnC R391 : S--TQTC NPQCI CKL ES-PIPIERLSRASA PASFSQA ATVGFRVTE TDRN AA SAQWNGKASRNPS : 196
 TrnC pCAR1 : I--SGVR EQSEV VTI AN-ALPIOTLANAAKE FDCWRTS DSCNLAAVA INDA REIPSE HOGGTASMRSDP : 208
 213 Rts1 : --VGVR DCLMC VKI IKANYEPKDELMNLFNSFTSFDTS GILGLAFSTRES DI ASVHGGENASWRDS : 201
 4264 pRS241d : V--IKALPHHRPF ACWLDGDA---DLSQKELKNFKRSYSVFGGEAY-VPHGDPDI KE GENHCRGLVIGDL- : 210
 TrnC F : GMNLPLT HYRPF YCS SKK---KSRADILEMEN VKIIRAS QGASITQTDQAQ DI GENHNNISLTPKR- : 224
 TrnC pSLT : GMNLPLT HYRPF YCA SRK---KSRADILEMEN VKIIRAS QGAYITQTDQAQ NI GENHNNISLTPKR- : 224
 TrnC R100 : GMNLPLT HYRPF YCS SKK---KSRADILEMEN VKIIRAS QGASITQTDQAQ DI GENHNNISLTPKR- : 225
 TrnC pNLI : D--APFY NTRFI VGAR SG---PIG-ADTILAS KDSLEGT QSLIPARDGPVEL AF DDECPAVNADKPE- : 199

TrnC R478 : FIDVNTMKPNE NVZGRKTY TENHFSST-----QSD-----ESZHRYPQLV V FVNVFGCMYELVV : 271
 TrnC R27 : HVEASTMRPNE NVZGRKTY TENHFSST-----QSD-----ESZHRYPQLV V FVNVFGCMYELVV : 271
 TrnC SXT : PIRSEADKIREVLDYDRAIK DQGLMLG-----DYVKTLEFK LDRINFCHGAASFAG : 234
 TrnC R391 : PIRSEADKIREVLDYDRAIK DQGLMLG-----DYVKTLEFK LDRINFCHGAASFAG : 234
 TrnC pCAR1 : DMKADTDKIREVLDYDRAIK DQGLMLG-----DYVKTLEFK LDRINFCHGAASFAG : 234
 213 Rts1 : PVQVQDILLSELDYDRAIK DQGLMLG-----DYVKTLEFK LDRINFCHGAASFAG : 234
 4264 pRS241d : --TYTAEANHLPAGGLT QRT-----AIGLMGEPMAVSSATIR FRENWQFVLGILLNGV : 267
 TrnC F : RQLDPYSDNYCVDSDFTLK RQYLTG-----LRDNGKNSTARILNFHIA NGLIAFWNSADNYS : 289
 TrnC pSLT : RQLDPYSDNYCVDSDFTLK RQYLTG-----LRDNGKNSTARILNFHIA NGLIAFWNSADNYS : 289
 TrnC R100 : RQLDPYSDNYCVDSDFTLK RQYLTG-----LRDNGKNSTARILNFHIA NGLIAFWNSADNYS : 290
 TrnC pNLI : --HYSELDDNYCVRDLETC TPDRIVLQTERFRTGERODGAPVCEVVDKFDWFFVRNLKQWAPWDVQKILC : 278

TrnC R478 : W HGRKT FSPF TO QPADPLKSKENVRVYAITNKQASIPSVVI C R PDMDNMTATREED AK-I RGYLTF : 351
 TrnC R27 : W HGRKT FSPF TO QPADPLKSKENVRVYAITNKQASIPSVVI C R PDMDNMTATREED AK-I RGYLTF : 351
 TrnC SXT : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 TrnC R391 : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 TrnC pCAR1 : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 213 Rts1 : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 4264 pRS241d : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 TrnC F : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 TrnC pSLT : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 TrnC R100 : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333
 TrnC pNLI : W TGSRG RGSF NV HTPSAEAMR RLET QWAVNQAYGP-ML V V AAKKKGDVFEAQE DR-P RANVT : 333

TrnC R478 : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC R27 : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC SXT : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC R391 : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC pCAR1 : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 213 Rts1 : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 4264 pRS241d : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC F : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC pSLT : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC R100 : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428
 TrnC pNLI : VMGS-NANSVQTAANLKE LESRVNADLS IVFVS SSCV MCND---PKT FDLRSEVVSNTGAALH FPGWK : 428

Walker A

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TrhC R478 : NTDRPV SLV REA MGLNKK---TSAS IVSATADGFWTAY NNYGA PRNNLVHYRSTTFKHLENEYD : 506
TrhC R27 : NTDRPV NL REA LGLNKK---TSAS IVSATADGFWTAY NNYGA PRNNLVHYRSTTFEGFKNNSYD : 506
TrAc SXT : G-TGTPV NFV RNK MSVS RD---SGS GCAAGGSGGFLVNE SLYLSE : 465
TrAc R391 : G-TGTPV NFV RNK MSVS RD---SGS GCAAGGSGGFLVNE SLYLSE : 465
TrhC pCAR1 : G-TGTHVAPFI RNK FVSFH RD---DTF AMAAAGSGGFLATNY TLYLSI : 478
213 Rts1 : G-TKNPA MLT DSA QMKN RD---DFT ACIAAE SGGFLTNY TAYRSL : 484
4264 pRS241d : GSGNGPG LLIGPGG MDKNA RD---SDG VAVGK SGGFLVNE TLYAA : 483
TrAc F : --LTPAG LAP YRN AFID FERGNT MA CGT SGGFLIQH RVLDS : 507
TrAc pSLT : --LTPAG LAP YRN AFID FERGNT MA CGT SGGFLIQH RVLDS : 507
TrAc R100 : --LTPAG LAP YRN AFID FERGNT MA CGT SGGFLIQH RVLDS : 509
TrAc pNL1 : G-GHIPH LLIGR GPFWSHON--AAG RVA FGR SGGFLVLOD CAAMGGA : 492

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TrhC R478 : DDDPDGAQVF R R DOG A CYNSGFD GKTDFTL AFIT ITVNDVFNAPFTGESTN AEDKDVQTI : 587
TrhC R27 : AFDPDGAQVF R R DOG S CYNSGFD GKKDFTL AFIT ITVGERVDFEATVNDGNNEDDDKDVQTI : 587
TrAc SXT : -----GQCW R R R R E K C VYD -EPLQ GDSGICL EIE YDE-----E-----A----- : 513
TrAc R391 : -----GQCW R R R R E K C VYD -EPLQ GDSGICL EIE YDE-----E-----A----- : 513
TrhC pCAR1 : -----AKVW R R R R L K S A K -CFAP QDSGICL EIE YSE-----E-----E----- : 526
213 Rts1 : -----AKVWC R R R R L K N C T G -DYL DDPKPKCL EIE YFG-----EDPTNTDGGNEE : 542
4264 pRS241d : -----GRVV R R R R K NTV ILG -TLG DGSVCIRL SM SAEDMEK---DEY-----RA---ATELVG : 543
TrAc F : -----GFAV R R R R D G K S C NMG ---VLDGETIRE ANIT IDOS-----AER----- : 553
TrAc pSLT : -----GFAV R R R R D G K S C NMG ---VLDGETIRE ANIT IDOS-----AER----- : 553
TrAc R100 : -----GFAV R R R R D G K S C NMG ---VLDGETIRE ANIT IDOS-----AER----- : 554
TrAc pNL1 : -----AKVI R R R R R E H VKARF -AFVE KLSGFSL DM DGEALAS---DED-----GEI---YEVECLA : 552

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TrhC R478 : MNLQ K1 SEKGLD D DQSV L L AEE QSRKRS RTGS TGFALRC XHE-----DK FLDGEGGAMC : 657
TrhC R27 : MNLQ K1 SEKGLD D DQSV L L SEE NESRKV RTGS TGFARRCSNHE-----DK FLDGDOGQMC : 657
TrAc SXT : MVLG AA AITOSTI RMAN KROTREL EIT---GAMLG AEA LNHE-----DR CHGEGYFET : 580
TrAc R391 : MVLG AA AITOSTI RMAN KROTREL EIT---GAMLG AEA LNHE-----DP CHGEGYFET : 580
TrhC pCAR1 : MVLG SA AITVPI D DTAG KRK TOC ETL---KTMID ADS LADP-----DP CHGHOFAT : 593
213 Rts1 : MVLG TV APOKQ D DTAG KRK ALLIRE---KOTTK AESCLKDA-----DP SHSSQYFET : 609
4264 pRS241d : MAAAGGEGKQSR EEFREGSRI ADV AEF---AAGEE WER LAFARE-----DC ANVVKLEAT : 612
TrAc F : --RDC SV SINGN DEVHEGL LQA PAS LAK---ENRARD VDF LNASDSEQAESPTIRS DEIVLDOGT : 630
TrAc pSLT : --RDC SV SINGN DEVHEGL LQA PAS LAK---ENRARD VDF LNASDSEQAESPTIRS DEIVLDOGT : 630
TrAc R100 : --RDC SV SINGN DEVHEGL LQA PAS LAK---ENRARD VDF LNASDSEQAESPTIRS DEIVLDOGT : 631
TrAc pNL1 : M KSI GO RDDR S TANG L DSA STV REX---QRHGT D AAL PALP-----SPYACHADA SHEL : 619

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Walker B

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TrhC R478 : EGSHVGR TDTLPP NFDSH FIE EEE KGTPT QTV MS QAA HA IMKDRG F L E MEY RPD SSGAG : 738
TrhC R27 : EGSHVGR TENLPP NFDSH FIE EEE KGTPT QTV MS QAA HA IMKDRG F L E MEY RPD ASGSS : 738
TrAc SXT : TQEBE H FNGHNN RFKNRFT EEE KGRKH QOV LQ YOI QD LGE DRP VFI EAD TQD VG--- : 657
TrAc R391 : TQEBE H FNGHNN RFKNRFT EEE KGRKH QOV LQ YOI QD LGE DRP VFI EAD TQD VG--- : 657
TrhC pCAR1 : SRDQ H FNGHNN RFKNRFT EEE KGRKH QOV LQ YOI QD LGE DRP VFI EAD TQD VG--- : 670
213 Rts1 : SRDQ H FNGHNN RFKNRFT EEE KGRKH QOV LQ YOI QD LGE DRP VFI EAD TQD VG--- : 670
4264 pRS241d : RQDP H FNGHNN RFKNRFT EEE KGRKH QOV LQ YOI QD LGE DRP VFI EAD TQD VG--- : 685
TrAc F : ANTDQYNSDEPS RDDAKMV EGGEDRPS LVA FSI IYI NPT RTP NLK K EGR R DFK HKVG- : 710
TrAc pSLT : ANTDQYNSDEPS RDDAKMV EGGEDRPS LVA FSI IYI NPT RTP NLK K EGR R DFK HKVG- : 710
TrAc R100 : ANTDQYNSDEPS RDDAKMV EGGEDRPS LVA FSI IYI NPT RTP NLK K EGR R DFK HKVG- : 711
TrAc pNL1 : KQET H FEGACS DLSAGLTF EEE ESKPERSVA TAT FLTRY RDL SVP T L L L GGGQMG--- : 696

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TrhC R478 : NQSNQFFSS LAA RFP TNCAG C EFE YIT---SSVGR LTAN D K IMKERS EAMKANKYFSTSDA EYE : 916
TrhC R27 : NQSNQFFSS LAA RFP TNCAG C EFE YIT---SSVGR LTAN D K IMKERS EAMKANKYFSTSDA EYE : 916
TrAc SXT : -----K I T G R F Y G S A T P N LYD---PTGY IAB ANMY G KAST INALKEGRLPLGEGGYE : 727
TrAc R391 : -----K I T G R F Y G S A T P N LYD---PTGY IAB ANMY G KAST INALKEGRLPLGEGGYE : 727
TrhC pCAR1 : -----E H O R F P Y G S A V P G N LYQ---NKVGC IAB A T E G KARA IDA IENKRLPLGEGGYR : 740
213 Rts1 : -----D I K G R F P Y K A A T P N LYK---DSVGR IAD A M E G GESA INEAGASKRLLDACYR : 755
4264 pRS241d : -----R I G V V F A Y T A L F G T E D Y N---NVAATVCLD T E A KART IDALVQDRRLVSPHIAG : 759
TrAc F : -----E I K G T A H T A Y T P N V DSDKASAAK AWG S K I K SAKFAKYNQLYPQDFPLQRD : 783
TrAc pSLT : -----D I K G T A H T A Y T P N V DSDKASAAK AWG S K I K SAKFAKYNQLYPQDFPLQRD : 783
TrAc R100 : -----E I K G T A H T A Y T P N V DSDKASAAK AWG S K I K SAKFAKYNQLYPQDFPLQRD : 784
TrAc pNL1 : -----QALTYA T Q Y G S L F A N N F Y K---SEGL ALD D S ECKERT INCLAKHORFEMDHYES : 766

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TrhC R478 : RKNIRIVK-KVEHML RFENFOEIC YV RKMELG T DSA RSKWE IQSPRENCYGEAIE-----LYQEVAAAG : 990
TrhC R27 : RKNIRIVK-RATHEML RFENFOEIC YV RKMELG T DST RSKWE IQSLDSCYGEAIE-----LYQEVASK : 990
TrAc SXT : YLTTHIVT-GVWEIFFITMG TGIG I FHL RAE VNA KULTRK L VAD SJ-----LKERGYE-- : 799
TrAc R391 : YLTTHIVT-GVWEIFFITMG TGIG I FHL RAE VNA KULTRK L VAD SJ-----LKERGYE-- : 799
TrhC pCAR1 : LNTVKYK-GHWEIF LTPRCACI V T Y I L I T A P E V O A R N Y T L V P Q E L R-----VLRDGV EYN : 814
213 Rts1 : FNTTRSS-GVWEIF ISKAGOGIS I T F L L V S E P D V S E R R K K D L T T E C Y I-----L R C G A A : 828
4264 pRS241d : QNSIQVVG-GLTHMG KGPNGWFFG L FSLA YAKGSTVERENRLKD YITVE R-----LVEGAVE- : 831
TrAc F : MEGKFGAAKDWTFSL QVNHSSWHEF L L R A V A D G P F E F Q O K R K E L I H E W L A N K S G P E A S L E A W : 864
TrAc pSLT : MEGKFGAAKDWTFSL QVNHSSWHEF L L R A V A D G P F E F Q O K R K E L I H E W L A N K S G P E A S L E A W : 864
TrAc R100 : MEGKFGAAKDWTFSL QVNHSSWHEF L L R A V A D G P F E F Q O K R K E L I H E W L A N K S G P E A S L E A W : 865
TrAc pNL1 : LRSIKRNG-TEHVL KGPETLAVG M Y S A T A S P R V F S E D K V R G L A L P D E R V A P D S T P E P A P D F : 846

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TrhC R478	:	LAA-----	:	893
TrhC R27	:	SAA-----	:	893
TraC SXT	:	-----	:	-
TraC R391	:	-----	:	-
TrhC pCAR1	:	PDPAEVA-----	:	821
213 Rta1	:	-----	:	-
4264 pRS241d	:	-----	:	-
TraC F	:	LEEHEKYRSVA-----	:	875
TraC pSLT	:	LQEHETFRGTYEYKAETD	:	882
TraC R100	:	LEEHEKYRSVA-----	:	876
TraC pNL1	:	AEGELAEAAE-----	:	856

Table 3-1. Plasmids and *E. coli* strains used in this chapter.

Strain or Plasmid	Description	Selective marker(s) ^b	Source
<i>E. coli</i> strain			
DH5 α	λ ϕ 80d/lacZ Δ M15 Δ (lacZYA-argF)U169 <i>recA endA1 hsdR17</i> (r _k ⁻ m _k ⁻)		Invitrogen
DH10B	F- <i>mrcA</i> Δ (<i>mrr-hsdRMA-mrcBC</i>) ϕ 80d/lacZ Δ M15 Δ lacX74 <i>deoR recA1 endA1 araD139</i> Δ (<i>ara, leu</i>) 7697 <i>galU galK</i> λ - <i>rpsL hupG</i>		Invitrogen
DY330	W3110 Δ lacU169 <i>gal490</i> λ c1857 Δ (<i>cro-bioA</i>); spontaneous Nal ^r or Rif ^r	Nal or Rif	Yu <i>et al.</i> , 2000
J53	<i>pro met</i> F ⁻	Nal	Bachman, 1972
JE2571	<i>leu thr str fla pil</i>	Nal or Rif	Bradley, 1980
RG192	<i>ara leu lac</i>	Rif	Taylor, 1983
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacZ</i> Δ m15 Tn10]	Tc	Stratagene
Plasmids			
pDT1942	Derepressed transfer mutant of R27	Tc, Km	Maher <i>et al.</i> , 1991
pDT2953	pDT1942 with mini::Tn10 inserted into <i>trhU</i>	Tc, Km, Cm	Rooker <i>et al.</i> , 1999
pDT2957	pDT1942 with mini::Tn10 inserted into <i>trhC</i>	Tc, Km, Cm	Taylor <i>et al.</i> , 1999
pDT2959	pDT1942 with mini::Tn10 inserted into <i>trhW</i>	Tc, Km, Cm	Rooker <i>et al.</i> , 1999
pDT2969	pDT1942 with mini::Tn10 inserted into <i>trhC</i>	Tc, Km, Cm	Taylor <i>et al.</i> , 1999
pDT2970	pDT1942 with mini::Tn10 inserted into <i>trhE</i>	Tc, Km, Cm	Rooker <i>et al.</i> , 1999
pDT2971	pDT1942 with mini::Tn10 inserted into <i>trhB</i>	Tc, Km, Cm	Rooker <i>et al.</i> , 1999
pDT2978	pDT1942 with mini::Tn10 inserted into <i>trhC</i>	Tc, Km, Cm	Taylor <i>et al.</i> , 1999
pDT2981	pDT1942 with mini::Tn10 inserted into <i>trhK</i>	Tc, Km, Cm	Rooker <i>et al.</i> , 1999
pDT2984	pDT1942 with mini::Tn10 inserted into <i>trhH</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pDT2987	pDT1942 with mini::Tn10 inserted into <i>trhF</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pDT2988	pDT1942 with mini::Tn10 inserted into <i>trhN</i>	Tc, Km, Cm	Rooker <i>et al.</i> , 1999
pDT2989	pDT1942 with mini::Tn10 inserted into <i>traG</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pDT2991	pDT1942 with mini::Tn10 inserted into <i>trhG</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pDT2995	pDT1942 with mini::Tn10 inserted into <i>tral</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pDT3048	R27 with <i>gfp/cat</i> inserted in-frame with <i>trhC</i>	Tc, Cm	Gilmour <i>et al.</i> , 2001
pGEM-T	Cloning vector for PCR products	Ap	Promega
pJEG51	pDT1942 with <i>cat</i> inserted into <i>traJ</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pJEG94	pDT1942 with <i>cat</i> inserted into <i>trhR</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pJEG104	pDT1942 with <i>cat</i> inserted into <i>traH</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pJEG115	pDT1942 with <i>cat</i> inserted into <i>trhY</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a
pGUN110	pDT1942 with <i>cat</i> inserted into <i>trhA</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2003a
pMS119EH	Cloning vector, <i>P</i> _{lac} - <i>lacF</i> ⁻ , pMB1 origin of replication	Ap	Strack <i>et al.</i> , 1992
pMWG36	TrhC _{HsdR} expression vector (pMS119EH)	Ap	Gilmour <i>et al.</i> , 2001
pMWG133	<i>gfp/cat</i> cloned into pGEM	Ap, Cm	Gilmour <i>et al.</i> , 2001
pMWG191	TrhC-GFP expression vector (pMS119EH)	Ap	Gilmour <i>et al.</i> , 2001
pMWG253	pDT1942 with <i>cat</i> inserted into <i>trhL</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2003a
pMWG265	pDT1942 with <i>cat</i> inserted into <i>trhV</i>	Tc, Km, Cm	Lawley <i>et al.</i> , 2003a
R27	IncHI1	Tc	Taylor, 1983

a. Antibiotic resistance markers: Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Nal, nalidixic acid; Rif, rifampicin

Hgal RNA bacteriophage was prepared as previously described (Maher *et al.*, 1991). To determine Hgal infectivity of TrhC mutants, phage spot tests were performed. A 50 μ L sample of mid-logarithmic phase culture was suspended in 8 ml of brain-heart infusion (BHI) broth (Difco) + 0.6% agar at 48°C. The suspension was immediately overlayed on a fresh BHI plate. When cultures contained a pMS119EH expression vector, ampicillin and IPTG were included in the overlay. After solidification of the overlay 10 μ L of Hgal stock (9.5×10^{10} PFU/ml) was spotted at discrete points on the overlay. Plates were incubated at 27°C and analyzed for zones of clearing in the phage-treated regions.

3.2.2 DNA and RNA techniques

The *gfp/cat* linear DNA cassette was constructed by recombinant PCR. First round PCR amplification of the green fluorescent protein gene *gfpmut2* (Cormack *et al.*, 1996) was achieved using primers GIL78 (5'-GATTCTGTACGAACAGGAAGTTGCGAGCAAATCAGCTGCCATGAGTAAAGGAGAAGAAC) and GIL79 (5'-GAAGTGATCTTCCGTCACAGTTATTTGTATAGTTCA TCCATG) and amplification of the Tn9 chloramphenicol acetyl transferase gene *cat* (including the native promoter) was with primers GIL80 (5'-CATGGATGAACTATACAAATAAC TGTGACGGAAGATCACTTC) and GIL81 (5'-ACCCGGGCTATTATTTCCGGGGAGGGCAGGCAATGACAGCTTATTCAGGCGTAGCACCAG). Each first round product was ethanol precipitated, re-dissolved in de-ionized water, and used as templates for a second round PCR to adjoin *gfp* and *cat* via overlap extension and amplification with the outside primers GIL78 and GIL81. The recombinant product was purified from an agarose gel using the Qiaquick gel extraction kit (Qiagen) and ligated into pGEM-T (Promega) to create pMWG133 and transformed into DH5 α . Clones containing the *gfp/cat* construct were selected for with Ap and Cm.

To insert *gfp* in-frame with the end of *trhC*, linear *gfp/cat* DNA was produced through PCR of pMWG133 using primers GIL78 and GIL81, purified using Qiaquick PCR purification columns (Qiagen), and electroporated into *E. coli* strain DY330(R27) following the procedure outlined by Yu *et al.* (Yu *et al.*, 2000). DNA was treated with *DpnI* (Life Technologies) prior to purification to remove remaining circular pMWG133. Recombinants were selected on Tc and Cm. To verify insertion of the *gfp/cat* cassette into R27 after the penultimate codon of *trhC*, clones were sequenced using primers GIL54 (5'-TGGTGCTACGCCTGAATAAG) and GIL88 (5'-AAGAATTGGGACAACTCCAGTG). Strain DT3048 contains R27::*trhC-gfp*. The *trhC-gfp* fusion product was amplified and subsequently cloned into pMS119EH (to create pMWG191) using the forward primer GIL20 (5'-TATGGTACCATGAGATCAGCTAACGTCTAT) and reverse primer GIL89 (5'-TATCTGCAGTTATTTGTATAGTTCATCCATG) and pDT3048 as the template DNA.

To clone *trhC* with a C terminal His₆-coding tag, the forward primer GIL20 the reverse primer GIL06 (5'-TATCTGCAGTCAGTGATGGTGATGGTGATGTGCGGCAGCTGATTGCTCGCAAC) were used. The PCR product was initially cloned into pGEM-T (Promega) and then subcloned into pMS119EH (see Table 3-1) using the restriction enzymes *KpnI* and *PstI* and T4 Ligase (Invitrogen).

Plasmids pMWG191 (encoding *trhC-gfp*) and pMWG36 (encoding *trhC_{His6}*) served as templates for site-directed mutagenesis of Walker A and B-encoding regions of TrhC. To introduce substitutions and deletions into *trhC*, a complementary pair of oligonucleotide primers containing mismatches or deletions in comparison to the template sequence were used in a thermocycling reaction with *PfuTurbo* (Stratagene) to synthesize full-length DNA molecules from the plasmid templates. These primers were based upon the following sequence from the *trhC* sense strand: 5'GCAACGTCTGGTGCTGG
TAAATCATTCTGGGTCGCATAC (Walker A) and 5'-CGGGTTGTTCACTTGATGAGGCATGGGAGT
ATATCCGTCCG (Walker B), where the underlined segments represent regions containing

mutation-specific mismatches or deletions (detailed below). The parental wild-type plasmid DNA was selectively destroyed using *DpnI* (Invitrogen), which recognizes and cleaves methylated DNA substrates. The newly synthesized, un-methylated DNA containing mutations was transformed into XL1-Blue *E. coli* ultra-competent cells (Stratagene) for circularization and propagation. The plasmid constructs were sequenced in the target area (using primers: Walker B: 5'-ACACCTTCAGACTGTCGTTCTG; Walker A: 5'-CTCCATCAGGGTCAAATGCATC) and analyzed by restriction digestion to ensure that mutated constructs of the proper size had been generated. All pMWG191 wild-type and mutation constructs were then transformed into RG192 cells containing pDT2969 for fluorescence assays, whereas pMWG36 wild-type and mutation constructs were transformed into RG192 cells containing pDT2957, pDT2969, or pDT2978 for conjugative transfer complementation and bacteriophage propagation assays.

For RT-PCR experiments, RNA was purified from 20 ml cultures of DY330(pDT3048) grown overnight at 27°C or 37°C. The cells were pelleted and then used in the GenElute Mammalian Total RNA Isolation Kit (Sigma), following manufacturer's directions. The resulting RNA was then quantitated with an Ultrospec 3000 (Pharmacia). The RNA was treated with DNase I (Invitrogen) to remove any residual DNA that could contaminate the sample. cDNA was made from the RNA using Superscript II reverse transcriptase (Invitrogen), following manufacturer's protocols. The primer used for cDNA synthesis, specific for *gfp* sequence, was GFP-rev (5'-TTTCGAAAGGGCAGATTG). The cDNA was then used in a PCR reaction with GFP-rev and the forward primer TrhC-RT-PCR (5'-ATTCCGGGTACACATTATTTCTA) specific to *trhC* sequence to amplify a product of 3230 bp. The PCR samples were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

3.2.3 Microscopy

For visualization of H-pili with the transmission electron microscope the method of Maher *et al.* (Maher and Taylor, 1993) was followed, with the exception that JE2571 cells were grown in LB broth, Lennox formulation (Difco).

For the fluorescence assay, *E. coli* cells were grown overnight in 20 ml LB broth containing antibiotics at either 27°C or 37°C. A 5% inoculum was transferred to 20 ml fresh LB-Lennox containing antibiotics (pre-equilibrated at either 27°C or 37°C) and incubation continued at that temperature with shaking until cells reached OD₆₀₀~0.4. A 1 ml aliquot was taken and the cells were pelleted in a microfuge. The supernatant was aspirated and cells were washed in 1 ml MOPS minimal media supplemented with 0.5% glucose and 20-amino acid mixture (Gordon *et al.*, 1997), pelleted and aspirated again. The cells were resuspended in a final volume of 50 µl of the above MOPS media. A 1 µl aliquot was spotted on a MOPS+glucose-1.5% agarose slab on a glass microscope slide. A cover slip was placed on and sealed, and the cells visualized using a UV light source through a Leica DMRE microscope equipped with a HQF filter and a Cooke SensiCam. Images were collected and processed using SensiControl 4.0 and PhotoPaint 8.0 (Corel) software.

3.2.4 Purification of TrhC and antibody production

Bacterial cultures of DH10B containing pMWG36 (4 litres) were grown at 30°C to OD₆₀₀~0.6 and induced with 0.5 mM IPTG for 1.5 hours. Cells were harvested by centrifugation (Beckman JLA 8.1 rotor, 15 minutes, and 7000xg) and resuspended in buffer A (20 mM Tris-HCl pH 7.6, 10% glycerol, and 0.5 M NaCl) with 50mM imidazole at a concentration of 3 ml/g of cells and 1 ml/20g of cells protease inhibitor cocktail (Sigma). Cells were lysed by three passages through a French press (16 000 p.s.i.) and the cytoplasm collected by centrifugation (Ti70:1 rotor, 100 000xg, 2 hours). The

supernatant was applied to 2 ml of Ni-NTA superflow resin (Qiagen) equilibrated in buffer A with 50 mM imidazole in a 1.5 x 10 cm Glass Econo-Column (Biorad). The column was washed three times with 5 ml of buffer A with 50 mM imidazole and TrhC was eluted with buffer A plus 200 mM imidazole. Fractions containing TrhC were concentrated with Centriprep-50 centrifugal concentrators (Amicon). Glycerol content was increased to 40% and the concentration of TrhC was estimated to be 2mg/ml. To further purify TrhC during antigen preparation, total protein (with addition of 100 mM Tris-HCl pH 7.6, 2% SDS, and 100 mM DTT) was loaded into a 10% SDS acrylamide gel. After electrophoresis the gel was washed three times in Milli-Q water then stained for ten minutes in 0.05% Coomassie stain. The gel was destained in Milli-Q water until the band corresponding to TrhC was discernable. This band was excised from the gel, air-dried, and ground to a fine powder with a mortar and pestle. The band isolated was verified to be TrhC by N-terminal sequencing through Edman degradation. The protein-acrylamide powder was resuspended in PBS until it reached the consistency to pass through a 21-gauge syringe, and then frozen until required for injection. Antibodies to TrhC were raised in New Zealand White rabbits. A primary inoculation of 1 mg of TrhC with complete Freund's adjuvant (Sigma) per rabbit was proceeded by a secondary inoculation on day 50 (0.5 mg) and a third injection on day 95 with another 0.5 mg, each with incomplete Freund's adjuvant. Production of TrhC-specific antisera was followed by immunoblot analysis. Proteins were resolved on 8% acrylamide gels, transferred to nitrocellulose (Micron Separations Inc.), and blocked overnight in 15% skim milk, PBS, and 0.1% Tween-20. Primary antibody was added at 1:1000 and secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG, Sigma) at 1:2000. Detection was by either the ECL kit (Amersham Pharmacia) or with the SuperSignal West Dura extended duration substrate (Pierce).

3.2.5 Purification of TrhC for enzymatic analysis

Bacterial cultures were grown and induced as described in section 3.2.4, and prior to lysis via the French press, cells were resuspended in 25 ml of buffer C (10mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 2.5 M NaCl) with 50 mM imidazole. TrhC was purified using Ni-NTA resin as described in section 3.2.4, except buffer C was used for washes (with 50 mM imidazole) and elution (7 ml with 350 mM imidazole). Protein content of the 1ml fractions was estimated using a standard Bradford solution assay, and three fractions containing the highest concentrations of protein were desalted using a PD-10 column (Amersham Biosciences) equilibrate with buffer D (50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂). Seven 1 ml fractions were collected and again analyzed for protein content using the Bradford assay. Protein-containing fractions were loaded into a MonoQ anion-exchange fast protein liquid chromatography (FPLC) column using buffer D at flow rate of 1 ml/min for 10 min. To elute bound proteins, a linear gradient of buffer E (1 M KCl) was used, starting at 0% and rising to 100% over the course of 20 min at a flow rate of 1ml/min. Protein content of was analyzed at 215 nm and 280 nm during elution and 1ml fractions were collected. An aliquot from each fraction was analyzed by SDS-PAGE.

3.2.6 ATPase assay

Reaction buffer (50 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 1 mM DTT) and ATP mix (1 mM cold ATP and 0.04 μ Ci ³²P- γ -ATP) were combined in 1.5 ml tube on ice. At time = 0, preparations of TrhC were added and the assay mixture (total volume, 100 μ l) was incubated at 28°C. At specific intervals, 30 μ l of the assay mixture was removed, added to 500 ml of stop solution (67% vol/vol 2-butanol, 133 mM H₂SO₄, 0.4 mM NaH₂PO₄, 13 mM MoNa₂O₄ - 2H₂O), and vortexed for 1 min. The organic phase containing cleaved γ -phosphates was recovered after centrifugation (5 min, 14000 g) and added to 5 ml of scintillation fluid. The radioactivity of each sample was quantified in a scintillation

counter calibrated for ^{32}P isotopes. To determine the background level of ATP hydrolysis in the liquid phase, assay mixtures containing only reaction buffer and ATP mix were incubated at 28°C for 30 min and processed as described above.

3.2.7 Localization of TrhC

To determine the approximate cellular location of TrhC, membrane isolation and fractionation as described by Grahm *et al.* (Grahm *et al.*, 2000) was performed. Using this procedure, cytoplasmic and whole membrane fractions were isolated. The membrane fraction was further separated into cytoplasmic and outer membrane fractions by isopycnic sucrose gradient centrifugation. 1 ml fractions collected from the bottom of the ultracentrifugation tube were analyzed for TrhC content by immunoblotting (see above).

3.3 Results

3.3.1 Insertion of *gfp/cat* into R27 to create a *trhC-gfp* fusion

In order to create a *trhC-gfp* fusion that would be expressed at the same rate and under the same conditions as native *trhC*, the fusion of *gfp* to *trhC* was performed using whole R27 plasmid DNA (Figure 3-2). The *gfpmut2* gene (Cormack *et al.*, 1996) was included as part of a linear DNA construct comprising the chloramphenicol acetyl transferase (*cat*) gene and terminal arms homologous to R27. Utilizing the λ prophage recombination system in the host strain *E. coli* DY330 (Yu *et al.*, 2000), the homologous arms directed recombination of the *gfp/cat* cassette into R27 at positions 28485-28639 to create pDT3048 (R27::*gfp/cat*, see Table 3-1). This resulted in a fusion construct encoding a C terminal fusion of GFP to TrhC.

The *trhC* gene was a candidate for *gfp* fusion due to the genetic organization of *trhC* in R27 and the predicted membrane orientation of TrhC. Knowing the membrane

Figure 3-2. Schematic diagram of *gfp/cat* cassette insertion into R27 to create a *trhC-gfp* translational fusion. The *cat* segment includes 197 bp upstream of the start codon and 54 bp downstream of the stop codon, therefore the native *cat* promoter is present.

topology of the VirB4 family of proteins, TrhC is predicted to be an inner membrane protein with the C terminal end of the protein remaining in the cytoplasm. Since GFP is not stable in the periplasm (Feilmeier *et al.*, 2000), a C-terminal fusion to TrhC is an appropriate choice to detect GFP fluorescence. In addition, the next open reading frame downstream of *trhC*, R0023, is 668 bp away, thereby allowing insertion of the *gfp/cat* construct without disrupting essential transfer genes.

The R27::*gfp/cat* construct pDT3048 was competent for plasmid transfer, albeit with a two-log decrease in transfer frequency compared to wild-type R27 (Table 3-2). *E. coli* DY330R carrying pDT3048 were resistant to lysis by the H-pilus-specific RNA phage Hgal, whereas *E. coli* carrying wild-type R27 were sensitive to Hgal, as measured in a phage spot assay (Table 3-2). In addition, no pili were observed on JE2571 cells carrying pDT3048 under transmission electron microscopy (data not shown). The lack of pili would result in the concurrent lack of RNA phage propagation, as there would be no structure to which the phage could adhere. The *gfp/cat* insertion was confirmed to have no polar effects as production of TrhC with a C terminal His₆ tag (pMWG36) *in trans* restored pDT3048 plasmid transfer frequency back to the wild-type level (Table 3-2). Using R27 with a transposon knockout mutant of *trhC* (pDT2969), the ability of TrhC-GFP to act in plasmid transfer was confirmed. Insertion of mini-Tn10 into *trhC* abolished transfer of R27, H-pilus production, and infectivity of Hgal, whereas expression of TrhC from pMWG36 complemented pDT2969 to wild-type transfer frequency and restored H-pilus production and Hgal propagation (Table 3-2). The *trhC-gfp* fusion was cloned into the pMS119EH expression vector (pMWG191) and expression of TrhC-GFP enabled pDT2969 to transfer, however at a lower frequency than achieved by TrhC_{His6}.

Furthermore, complementation with TrhC-GFP was unable to restore Hgal propagation and no pili were detected (Table 3-2). Therefore, the fusion of GFP to TrhC still supports R27 plasmid transfer with both an *in situ* R27 fusion and during *trans* complementation

Table 3-2. Conjugative transfer, RNA phage propagation, and H-pilus production by R27 and derivatives

Plasmid(s) in the donor strain	Transfer frequency ^a	Propagation of Hgal ^b	Production of H-pilus ^c
R27	3.4×10^{-1}	+	+
pDT3048	1.3×10^{-3}	-	-
pDT3048 + pMWG36	2.8×10^{-1}	+	+
pDT2969	0	-	-
pDT2969 + pMWG36	8.3×10^{-1}	+	+
pDT2969 + pMWG191	1.5×10^{-5}	-	-

a. transconjugants/donor, 18 hour matings

b. as determined in a agar plate spot test; "-" indicates no zones of lysis were observed

c. as determined in the transmission electron microscope; "-" indicates no pili were observed on the examined cells

of a *trhC* knockout, but the proficiency of TrhC-GFP to fulfill roles in pilus production, phage entry, and conjugation is reduced. It seems likely that TrhC-GFP is maintaining association with the conjugative apparatus because plasmid transfer is still occurring.

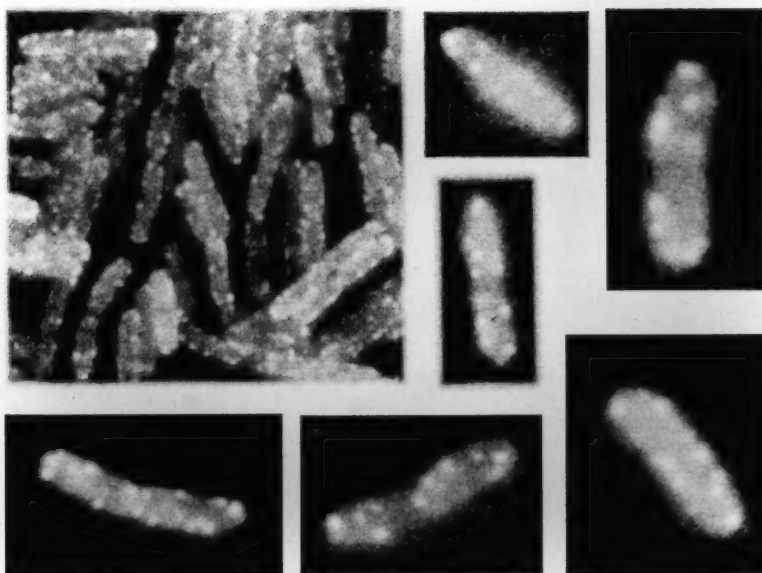
3.3.2 Localization of TrhC to the cytoplasmic membrane

GFP labeling of both DNA and protein have been used as a tool for subcellular localization. The LacI-GFP system has been used to track the segregation of bacterial chromosomes (Gordon *et al.*, 1997) and conjugative plasmids (Pogliano *et al.*, 2001). Fusion of GFP to the DNA replication protein PolC in *Bacillus subtilis* allowed determination of distinct sites for the DNA replication machinery (Lemon and Grossman, 2000). Under fluorescence microscopy, *E. coli* cells carrying pDT3048 contained discrete fluorescent foci primarily at the cell periphery, implying a membrane association of TrhC (Figure 3-3A).

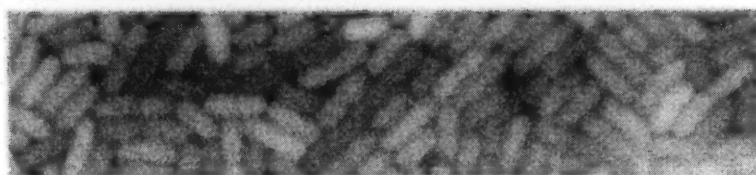
R27 has recently been shown to segregate to and maintain a distinct subcellular localization at the quarter cell (1/4 and 3/4) and mid cell positions (Lawley, 2003). To determine if there could be a correlation between the cellular location of R27 and the cellular location of the R27 transfer protein TrhC, the relative cellular position of TrhC-GFP was examined through spatial analysis of the GFP foci in 118 cells grown at 27°C. There was no evidence that TrhC-GFP co-localizes to these plasmid domains. Each cell was divided into six equal portions across the lateral surface and individual foci cataloged into one of the six segments. The first and last segments represented the polar regions and a combined one-third of the entire cell length. Likewise, the second and fifth segments were combined to represent the quarter cell position, and the third and fourth segments added together and designated as the mid cell position, where each of these positions also represents one-third of the entire cell length. There was an average of 6 discrete fluorescent foci per cell with 39% of foci found in the polar

Figure 3-3. Fluorescent visualization of *Escherichia coli* DY330R(pDT3048) at 27°C and 37°C. Bacteria were grown exclusively at either 27°C (panel A) or 37°C (B) and visualized under UV illumination.

A



B



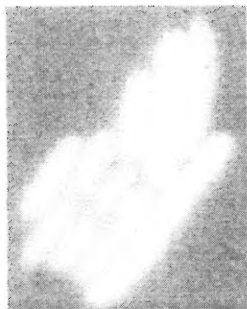
positions, 32% in the quarter cell positions, and 29% in the mid cell position. Since it is unlikely a single GFP molecule would be detected in our system, TrhC-GFP therefore seems to be present in protein complexes which are randomly distributed throughout the bacterial cell wall, with a moderate bias for the polar regions. Additionally, the location of pili on the outside of cells carrying R27 or TrhC-complemented pDT3048 appeared to be random, as observed during electron microscopy (data not shown).

When *trhC-gfp* expression was placed under control of the P_{tac} promoter (pMWG191) there was a uniform pattern of GFP fluorescence at both 37°C and 27°C (Figures 3-4A and 3-4B, respectively). Cells fluoresced with an entirely homogeneous and ubiquitous pattern: there were neither discrete foci nor a distinguishable subcellular localization pattern for the fusion protein. When pMWG191 was transformed into *E. coli* RG192 containing the R27 transfer mutant pDT2969 (see Table 3-1), cells still fluoresced with a homogeneous pattern when grown at 37°C (Figure 3-4C). However, when grown at 27°C, fluorescent foci were visualized at the cell periphery (Figure 3-4D). These data imply that R27-encoded proteins are required for the formation of membrane-associated fluorescent foci of TrhC-GFP, and this process is temperature-dependent.

E. coli cells expressing TrhC (pMWG36) and TrhC-GFP (pDT3048) were separated into whole cytoplasm and membrane fractions. The total membrane fraction was then further resolved into outer and cytoplasmic membrane fractions by isopycnic sucrose gradient centrifugation and the TrhC content of each fraction was assessed by immunoblot analysis with anti-TrhC antibodies (Figure 3-5). Membrane separation was confirmed by SDS-PAGE (data not shown) and density measurement of individual sucrose fractions. TrhC expressed from pMWG36 localized to both the cytoplasm and membrane, and within the latter preparation, entirely to the cytoplasmic (inner) membrane in sucrose fractions of 1.14g/cm³. The cytoplasmic TrhC may represent unsedimented membrane vesicles containing TrhC or an artifact of over-expression.

Figure 3-4. The formation of fluorescent foci is dependent upon the presence of R27-encoded proteins. DH5 α (pMWG191) grown at 37°C (panel A) or 27°C (B) and RG192(pMWG191+pDT2969) grown at either 37°C (C) or 27°C (D) were visualized under UV illumination. Cultures were not induced with IPTG.

A



B



C



D

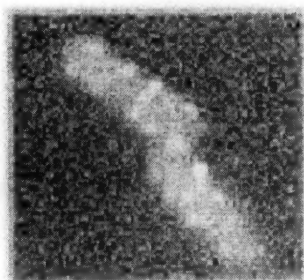
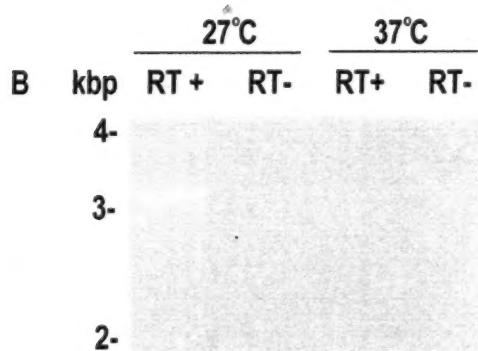


Figure 3-5. TrhC and TrhC-GFP localization and temperature-dependent expression.

Whole cell lysates (WC), total cytoplasmic fractions (CYT), total membrane fractions (MEM), outer membrane fractions (OM), and cytoplasmic membrane fractions (CM) prepared from cells expressing TrhC (pMWG36) and TrhC-GFP (pMWG191 and pDT3048) were probed with anti-TrhC polyclonal antibody after SDS-PAGE and transfer to nitrocellulose. All cells were grown at 37°C unless indicated (panel A).

DY330R(pDT3048) was grown at either 27°C or 37°C and mRNA was isolated for RT-PCR experiments specific for *trhC-gfp* mRNA (B). PCR reactions were performed on cDNA templates prepared in the presence (RT+) or absence (RT-) of reverse transcriptase.

A



Since these microscopic studies involved examination of TrhC-GFP, it was also appropriate to determine into which membrane the GFP fusion protein inserted. TrhC-GFP, expressed from pDT3048, was found in the cytoplasmic membrane in sucrose fractions of 1.15g/cm³ (Figure 3-5). These results confirm that both TrhC and TrhC-GFP are components of the cytoplasmic membrane.

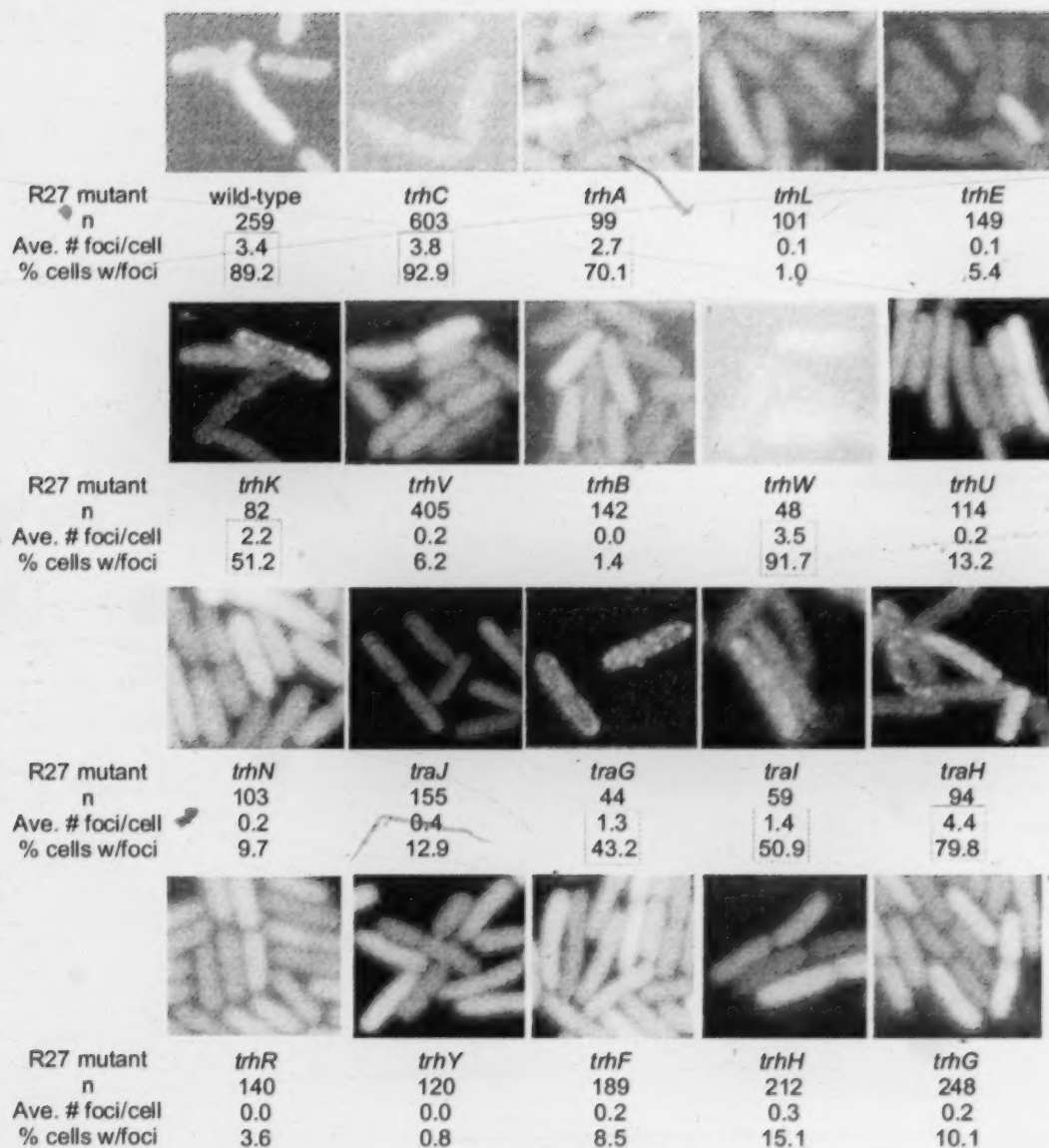
3.3.3 Specific R27 transfer proteins are required for assembly of the TrhC-GFP complex

To verify that the fluorescent foci visualized in cells with pDT3048 (or pDT2969 + pMWG191) result from an interaction between TrhC-GFP and other transfer proteins, TrhC-GFP was produced in the presence of R27 transfer mutants. The key observation relating to this experiment is the dependency of foci formation on the presence of R27-encoded proteins (see Figure 3-4). This implied that R27-encoded proteins were responsible for foci formation, and were likely part of a protein complex containing enough TrhC-GFP molecules to be visible.

Our group have previously generated transfer mutants in two separate regions of R27 encoding transfer functions (Tra1 and Tra2) by transposon and site-directed mutagenesis (Table 3-1), and in this study each of these transfer-deficient mutants were used in conjunction with pMWG191 to evaluate the requirements for TrhC-GFP to enter into membrane-associated protein complexes. Notably, each of the R27 transfer mutants have previously been complemented for transfer by *in trans* production of the respective wild-type transfer protein (Lawley *et al.*, 2002a; Lawley *et al.*, 2003a), therefore extreme polar effects caused by the gene disruptions were not observed.

After separately transforming the *trhC-gfp* expression plasmid pMWG191 into *E. coli* cells containing one of the nineteen different R27 transfer mutants, preparations of live cells were visualized under UV illumination (Figure 3-6). Twelve of the nineteen R27 transfer mutant were unable to support formation of fluorescent foci, as these cells

Figure 3-6. Fluorescence microscopy of live *E. coli* cells harbouring pMWG191 and a R27 transfer mutant. "n" cells were evaluated for the presence of fluorescent foci under the same criteria as previously described in the Experimental procedures. Solid boxes indicate cells having fluorescence patterns similar to R27 + pMWG191, while broken-line boxes indicate fluorescence patterns having foci, but not to the full extent as the positive control.



contained on average between 0.0 and 0.4 foci per cell, and the percentage of cells containing ≥ 1 focus ranged from 0.8%-15% among the different mutants (Figure 3-6). In these instances where a particular mutation prohibits foci formation, the missing transfer protein is probably a component of the TrhC-GFP complex or is directly required for its assembly. The loss of these requisite components likely results in incomplete (i.e. transfer-deficient) subassemblies that are below the threshold level of visualization.

In cells containing pMWG191 and R27 transfer mutants of *trhC*, *trhA*, *trhK*, *trhW*, *traG*, *tral*, or *traH* (Figure 3-6), fluorescent foci were observed. The average number of foci per cell ranged from 1.3 to 4.4, whereas the percentage of cells containing foci ranged from 43% - 93% among these mutants (Figure 3-6). These genes, with the exception of *trhC*, are not strictly required for the assembly of fluorescent foci and, in particular, cells lacking TrhW (predicted inner membrane Mpf protein) or TraH (predicted relaxosomal component) demonstrated no reduction in foci. In contrast, intermediary effects on the formation of foci were observed in cells lacking TrhA (pilin subunit), TrhK (predicted outer membrane secretin), TraG (inner membrane coupling protein), and Tral (DNA relaxase). These results suggest that several of the essential conjugative transfer proteins are not required for focus formation. The exact composition of the TrhC-GFP protein complex was not obtained in these experiments, and each of the above six transfer proteins may still be components of the TrhC-containing complex, but are not absolutely required for its assembly. The intermediate effects seen with four transfer mutants might have been elicited by unknown consequences of each mutation (including the altered stability of other essential transfer proteins).

In this fluorescence assay, TrhC is produced in two forms: TrhC-GFP (from pMWG191) and TrhC (from all R27 transfer mutants except the *trhC* mutant). To determine the effect of co-production of the two TrhC species for focus formation, cells carrying pMWG191 and wild-type R27 were examined (Figure 3-6). The fluorescence

patterns of these cells was similar to cells carrying pMWG191 and a R27 *trhC* mutant, therefore the presence of native TrhC does not affect TrhC-GFP condensation into foci. Furthermore, the over-production of TrhC-GFP from pMWG191 results in approximately a two-log imbalanced stoichiometry between the fusion protein and the other transfer proteins (our unpublished observations). The excess TrhC-GFP in cells containing foci was likely seen as the confluent (non foci-associated) fluorescence, similar to that seen in cells lacking R27 but producing TrhC-GFP.

3.3.4 Temperature-dependent mechanisms of R27 transfer

The transfer of IncHI plasmids is temperature-sensitive, as conjugation proceeds between 22-30°C, but not at 37°C. The temperature of the donor cells prior to and during mating is critical, not the temperature of the recipient (Taylor and Levine, 1980). The mechanism(s) underlying this phenomenon has not been revealed. Peter Newnham has previously examined the temperature-dependent kinetics of IncHI1 plasmid transfer by determining the effect of high temperature on mating frequency (Newnham, 1995). After pulsing mating mixtures at 37°C for various times, a direct negative logarithmic relationship existed between the time of exposure at 37°C and extent of DNA transfer, with a transfer frequency reduction rate of approximately 27% per minute pulsed at 37°C (Newnham, 1995). This suggests that exposure to 37°C causes a rapid and prolonged suppression of the transfer system of R27. This rapid response indicates that a pre-formed component in the transfer apparatus is immediately affected by the temperature shift.

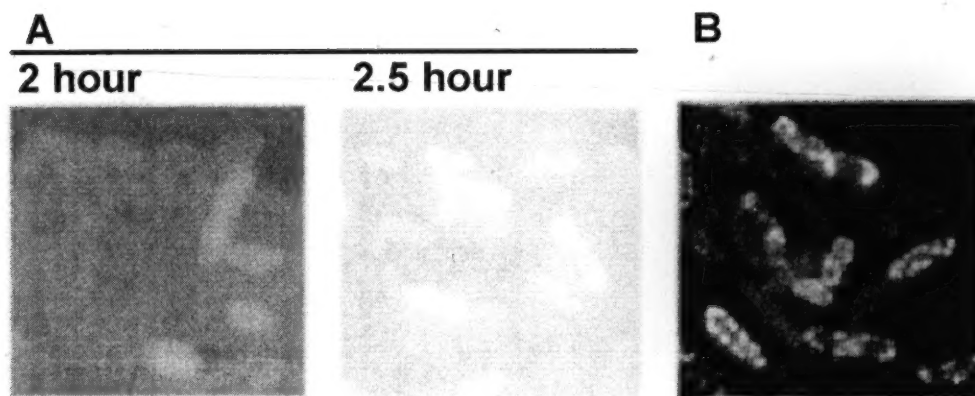
The temperature-dependent synthesis of TrhC was examined using the *trhC-gfp* fusion R27 construct pDT3048. Cultures containing pDT3048 that were grown exclusively at 27°C (Figure 3-3A) showed a marked difference in the level of fluorescence compared with cells grown at 37°C (Figure 3-3B). The majority of the cells

grown at 37°C had no detectable GFP fluorescence, as only a small proportion of cells contained GFP foci (<1%). Alternatively, the cells grown exclusively at 27°C almost all contained GFP foci. It is therefore apparent that a 10°C increase in incubation temperature dramatically alters *trhC-gfp* expression and/or the stability of TrhC-GFP.

To differentiate between two possibilities underlying temperature-sensitive transfer (transfer protein instability or repressed transfer protein production at 37°C) the fluorescence pattern of TrhC-GFP expressed from pMWG191 (under control of the P_{tac} promoter) at 37°C was examined (Figure 3-4A). The fusion protein fluoresced with same ubiquitous pattern as observed when expressed at 27°C (Figure 3-4B). The fusion protein is therefore stably maintained at the elevated temperature. The temperature-dependent production of TrhC-GFP protein and mRNA was confirmed by immunoblot analysis (Figure 3-5A) and RT-PCR (Figure 3-5B). Neither TrhC-GFP protein or mRNA could be detected in DY330R(pDT3048) grown at 37°C, but was found when cells were cultured at 27°C. These results support a model in which the synthesis of TrhC is temperature-sensitive, likely at the level of transcription.

To further reveal the kinetics of TrhC-GFP expression from pDT3048, temperature shift experiments were conducted. DY330R(pDT3048) was grown at 37°C to mid-logarithmic phase, therefore having little to no TrhC-GFP expression, and then equilibrated to 27°C in a water bath. Incubation (with shaking) was then continued at this lower temperature and samples were taken every half hour after the switch and processed as described (section 3.2.3). On average, 2.5 hours were required for regeneration of GFP foci (Figure 3-7A). Conversely, during shifts from 27°C to 37°C for 60 minutes, the GFP foci remained stable (Figure 3-7B). It appears that TrhC is not the transfer element which is immediately affected by high temperature, but that a considerable lag period is required for TrhC-GFP synthesis after growth at 37°C.

Figure 3-7. Temperature-dependent synthesis of TrhC-GFP foci and stability of TrhC-GFP foci at 37°C. *Escherichia coli* DY330R(pDT3048) was grown at 37°C until mid-logarithmic growth phase, cooled to 27°C (t=0 hour) and incubated at 27°C with shaking. Samples taken at the given time points were visualized using fluorescent microscopy (panel A). DY330R(pDT3048) was grown at 27°C, pulsed at 37°C for one hour, re-equilibrated to 27°C then processed for fluorescent microscopy (B).

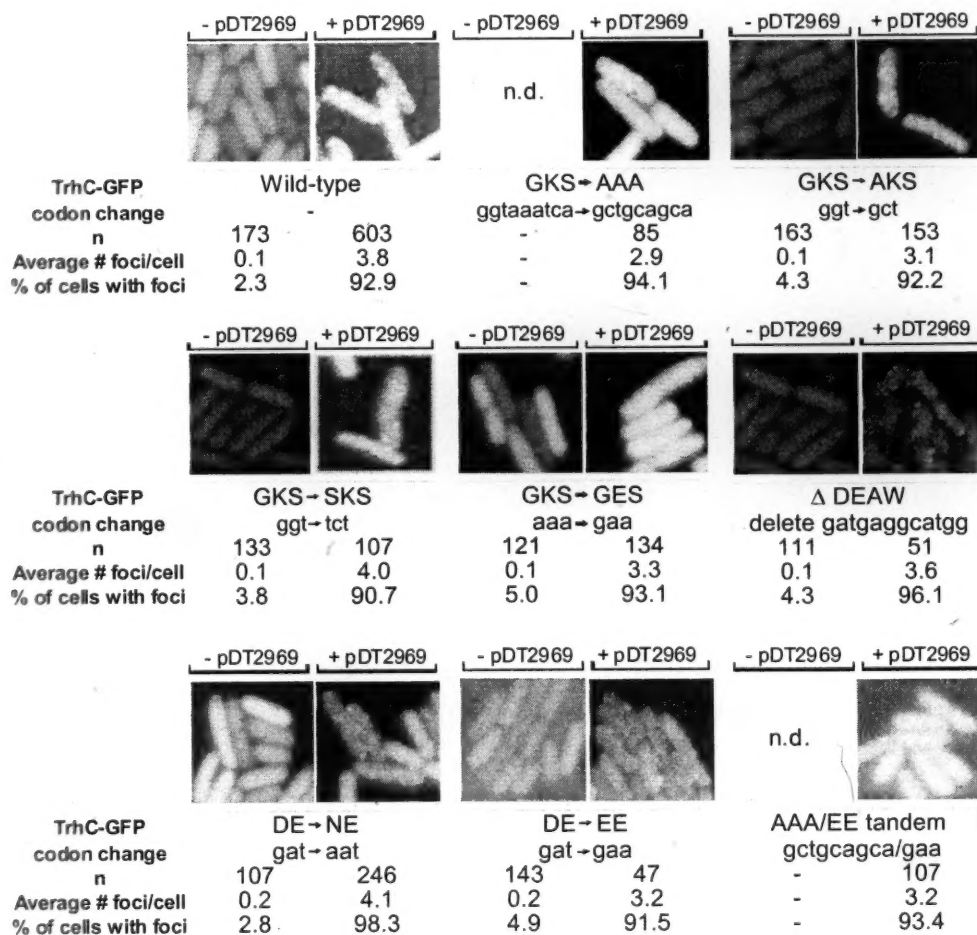


3.3.5 Mutations in the NTP-binding motifs of TrhC do not affect fluorescent foci formation

After site-directed mutagenesis using primers to introduce deletions and substitutions in the Walker A and B motif-encoding regions of *trhC* (pMWG36) and *trhC-gfp* (pMWG191, Figure 3-8), the resulting plasmid constructs were transformed into both DY330R cells and DY330R cells containing pDT2969 (a R27 *trhC* transfer mutant). In the Walker A motif (Figure 3-1), mutations were introduced in the region encoding Gly-Lys-Ser (GKS). These include substitution of all three residues to the small, non-polar amino acid alanine (GKS to AAA), a single Ala substitution of the similarly small, non-polar Gly (AKS), Gly substituted to a small, polar Ser (SKS), and the more dramatic substitution of Lys (positively charged, polar) to Glu (acidic side chain, polar; GES). In the Walker B motif the Asp-Glu-Ala-Trp sub-region (DEAW; Figure 3-1) was deleted; and also substituted the highly conserved charged polar residues Asp or Glu (DE sub-region) with the uncharged Asn (NE) and Glu (EE). A tandem substitution in both Walker A and B motifs (AAA/EE) was created using two successive rounds of primer-mediated site-directed mutagenesis.

In the fluorescence assay (Figure 3-8), production of the TrhC-GFP fusion proteins (wild-type or NTP-binding motif mutations) in cells lacking pDT2969 resulted in confluent, uniform fluorescence. For any of these trials in which the complement of R27-encoded proteins was absent, the average number of foci per cell was ≤ 0.2 and the percentage of cells having one or more foci was $\leq 5.0\%$ (Figure 3-8). In contrast, production of TrhC-GFP (wild-type or NTP-binding motif mutations) within cells containing pDT2969 resulted in an average of ≥ 2.9 foci per cell and $\geq 91\%$ of cells having one or more foci (Figure 3-8). When the extent of foci present in cells producing wild-type TrhC-GFP versus mutant TrhC-GFP were compared, little difference was observed.

Figure 3-8. Fluorescence microscopy of live *E. coli* cells harbouring pMWG191 or derivatives encoding mutations in Walker A or B motifs; with (right panels) or without (left panels) pDT2969. "n" cells were evaluated for the presence of fluorescent foci under the same criteria as described in Experimental procedures. "n.d" is not determined.



These data indicate that the NTP-binding motifs are not required for the assembly of TrhC-associated transfer protein complexes.

3.3.6 Mutations in the NTP-binding motifs of TrhC prohibit plasmid transfer

To determine the effect of NTP-binding motif mutations on conjugative transfer, standard conjugative transfer complementation experiments were conducted. The ability of pMWG36 and derivatives to restore, *in trans*, the transfer of three different R27 *trhC* transfer mutants was tested (Table 3-3). Production of wild-type TrhC from pMWG36 restored transfer of pDT2957, pDT2969, and pDT2978-containing donor cells to wild-type R27 transfer levels ($\sim 10^{-1}$ transconjugant/donor under these conditions, data not shown). All mutated pMWG36 constructs had zero or severely impaired ($>10^6$ -fold reduction) complementation of pDT2957 and pDT2978-containing cells (Table 3-3). The tandem mutation of both Walker A and B motifs also did not complement transfer, and cumulatively, these results suggest that the NTP-binding motifs of TrhC are required for conjugative DNA transfer. Comparable results were also found during mutational studies of VirB4 from the Ti plasmid (Berger and Christie, 1993) and the related TrbE from IncP plasmid RP4 (Rabel *et al.*, 2003).

Complementation of pDT2969 with Walker A mutation constructs of pMWG36, in comparison with the complementation results with wild-type pMWG36, resulted in greater than 10^4 -fold reduction in transfer frequency (Table 3-3). The insertion of mini-Tn10 into the *trhC* gene of pDT2957 and pDT2978 occur upstream of the Walker A-encoding region, whereas in pDT2969 mini-Tn10 is inserted downstream of the Walker A sequence, but upstream of the Walker B sequence (Taylor *et al.*, 1999). It is possible that a truncated TrhC peptide containing an unaltered Walker A motif is produced from pDT2969 which can weakly support transfer when full-length, albeit mutated, TrhC is supplied *in trans*. TrhC-like peptides might also be produced from pDT2957 and

Table 3-3. Complementation of conjugative transfer and susceptibility to bacteriophage of donor cells containing i) R27 *trhC* transfer mutants, and ii) pMWG36 or derivatives encoding TrhC Walker motif mutations. Underlined residues indicated amino acid substitutions in sub-regions of the Walker A (GKS) and/or Walker B (DEAW) motifs encoded in the pMWG36 derivatives. Units of transfer are transconjugants/donor. A transfer frequency of "0" refers to the absence of colonies on plates selecting for transconjugants, whereas "<10⁻⁷" refers the presence of a small amount of transconjugant colonies that lay outside the dilution range of the experiment to accurately define a transfer frequency. The susceptibility to the H-pilus specific bacteriophage Hgal was determined in phage spot assays, and lysis was scored as "+", whereas resistant strains were scored as "-".

R27 pMWG36	pDT2957		pDT2978		pDT2969	
	transfer	phage	transfer	phage	transfer	phage
Wild-type	8.3×10^{-2}	+	2.3×10^{-1}	+	2.4×10^{-1}	+
<u>AAA</u>	0	-	0	-	9.7×10^{-5}	-
<u>AKS</u>	0	-	0	-	6.0×10^{-5}	-
<u>SKS</u>	0	-	0	-	7.7×10^{-5}	-
<u>GES</u>	0	-	0	-	3.6×10^{-5}	-
del. DEAW	0	-	< 10 ⁻⁷	-	0	-
<u>NE</u>	0	-	0	-	0	-
<u>EE</u>	< 10 ⁻⁷	-	< 10 ⁻⁷	-	1.6×10^{-4}	-
<u>AAA/EE</u>	0	-	0	-	7.2×10^{-7}	-

pDT2978, but considering the point of mini-Tn10 insertion in these plasmids (Taylor *et al.*, 1999), no peptides containing Walker A or B motifs would likely be produced.

It is also possible that recombination between pMWG36 mutants and pDT2969 occurred, generating a self-transferable form of pDT2969 which resulted in the observed transfer events. To rule out this possibility, transconjugants from each pMWG36 mutation construct + pDT2969 complementation experiment were used as donors. No transconjugants were detected in this subsequent mating (data not shown), suggesting that transconjugants from the primary mating received intact pDT2969, and not a recombinant form containing a functional copy of *trhC*.

Complementation of pDT2969 with Walker B mutation constructs of pMWG36 gave variable results (Table 3-3). The deletion of amino acids DEAW or substitution of Asp to Arg did not support transfer, whereas a substitution of Asp to Glu decreased transfer only ~1000-fold. The capability of the Asp-Glu substitution to partially complement transfer may result from the subtle biochemical difference between these two amino acids (the chain length of Asp and Glu R-groups differ by one CH₂ group).

3.3.7 Mutations in the NTP-binding motifs of TrhC prohibit phage uptake/propagation

Another method used to determine the functionality of *trhC* clones encoding NTP-binding motif mutations was the susceptibility of donor cells to a pilus-specific bacteriophage that lyses cells with functional Mpf determinants. Using Hgal, a bacteriophage specific for IncH plasmid-containing cells (Maher *et al.*, 1991), the donor cells harboring pDT2957, pDT2969, or pDT2978 + pMWG36 or pMWG36-derivatives vulnerable to this RNA phage were determined (Table 3-3). For those cells containing wild-type pMWG36 and any of the three R27 transfer mutants, there was significant lysis, whereas cells containing any of the pMWG36 constructs encoding mutations in the

trhC Walker motifs and any of the three R27 *trhC* transfer mutants were resistant to HgaI (Table 3-3). Phage susceptibility depends on the Mpf proteins, and the inability of *TrhC* NTP-binding motif mutants to support this function (or conjugative transfer) indicates that these are critical domains for the overall functions supplied by *TrhC*.

3.3.8 Production and stability of *TrhC* and *TrhC*-GFP

Mutations made in the Walker A and B motifs of *TrhC* and *TrhC*-GFP, and the expression of *TrhC*-GFP in cells containing different complements of transfer proteins could result in altered stability of *TrhC* and *TrhC*-GFP. If true, insufficient amounts of *TrhC* protein may be present to support complementation of conjugative transfer or the formation of fluorescent foci. To determine the level of intact *TrhC* or *TrhC*-GFP produced in each strain used during fluorescence, complementation, and bacteriophage susceptibility assays, whole cell lysates were probed using anti-*TrhC* polyclonal sera (Figure 3-9). In all circumstances, either *TrhC* or *TrhC*-GFP was detected in cells carrying R27 transfer mutants and pMWG36 or pMWG191. The predominant species detected in each lysate correlated to whole *TrhC* or *TrhC*-GFP, and few degradation products were observed. These data suggest that the reduced transfer frequency of R27 when supported by *TrhC* Walker A or B mutants resulted from alteration of the NTP-binding domain, rather than decreased stability of the mutants. Additionally, the stability of *TrhC*-GFP was not drastically influenced by the absence of individual transfer proteins; therefore the deficiency in foci production with twelve R27 transfer mutants is likely due to the necessity of those transfer proteins for protein complex assembly, rather than *TrhC*-GFP degradation in their absence.

3.3.9 Enzymatic analysis of *TrhC*

TrhC and *TrhC* Walker mutants were purified from *E. coli* lysates using a combination of immobilized metal affinity chromatography and anion-exchange fast liquid

Figure 3-9. Production and stability of TrhC and TrhC-GFP. Polyclonal TrhC antisera was used to detect TrhC or TrhC-GFP in whole lysates of IPTG-induced cultures harbouring (A) wild-type or mutant pMWG36 or pMWG191 + pDT2969, or (B) pMWG191 + R27 transfer mutants. Underlined residues indicated amino acid substitutions in sub-regions of the Walker A (GKS) and/or Walker B (DEAW) motifs. "-" represents the whole cell lysate of RG192 + pDT2969 used as a negative control. Approximately equal amounts of total protein were loaded from each lysate, as the amount of sample used was equalized using optical density measurements of each culture prior to sample preparation, and verified using Coomassie-stained SDS-PAGE (data not shown).

A. pMWG36 or pMWG191 mutants and pDT2969

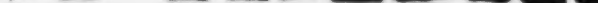
(WT)									AAA
GKS	AAA	AKS	SKS	~~GES~~	~~IDEAW~~	NE	~~EE~~	~~EE~~	.

TrhC_{H56} >

TrhC-GFP>

B. pMWG191 and drR27 transfer mutants

trhC - *trhE* ~~*trhK*~~ *trhB* *trhW* *trhN* *trhF* ~~*trhG*~~ *tral* *traG*

TrhC-GFP> 

trhA *trhL* *trhV* ~~*trhU*~~ *trhH* *traJ* *traH* *tirR* *trhY*

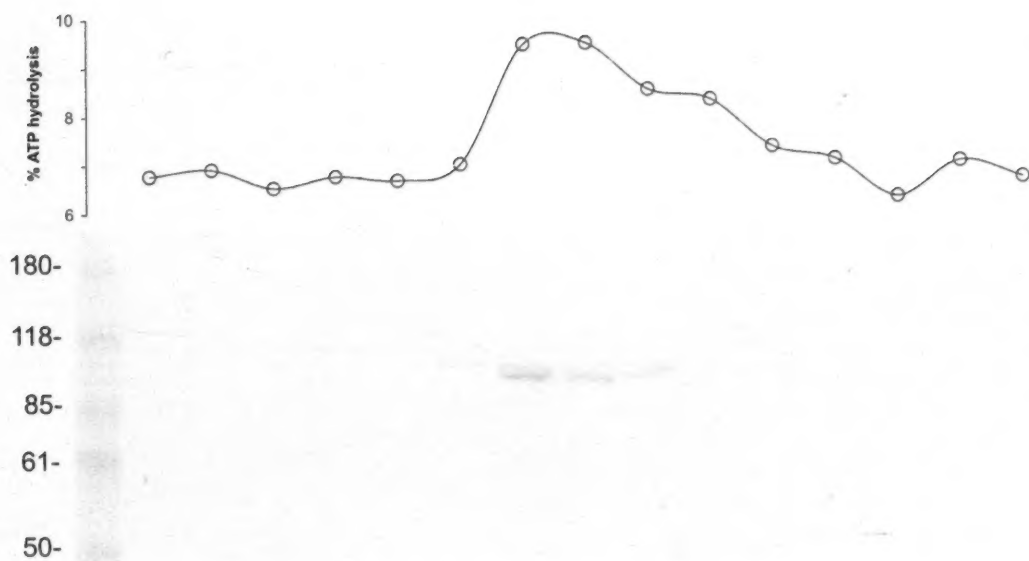
TrhC-GFP>

chromatography. No detergents were required to isolate TrhC from the bacterial membrane, as a high concentration of salt was sufficient for isolation. To approximate the amount of ATPase activity of the purified TrhC preparations, aliquots from the protein-containing fractions from the FPLC were incubated with ^{32}P - γ -ATP and excess cold ATP. Inorganic phosphate (the product of an ATPase reaction) was extracted with butanol, and the amount of radioactivity in this organic phase was determined. Purified wild-type TrhC and the subsequent enzymatic analysis is shown in Figure 3-10. The rise in apparent ATPase activity correlates with TrhC-containing fractions, however, the same activity profile was seen with preparations of TrhC containing a Lys to Glu mutation in the Walker A box (data not shown). This implies that the small amount of observed ATPase activity for these fractions is due to contaminating proteins, rather than the TrhC species because Lys residue has been shown to be essential for function of TrhC and other related proteins (Berger and Christie, 1993; Rabel *et al.*, 2003; Schneider and Hunke, 1998). A contaminating protein of approximately 60 kDa is visible in the seventh fraction shown in Figure 3-10, which also is the fraction having the highest ATPase activity. This could possibly represent a metal ion-binding protein that co-purified with TrhC during affinity chromatography using Ni-NTA resin.

3.4 Discussion

The R27 conjugative transfer system encodes sixteen Mpf proteins and a coupling protein which are presumed to assemble into a transmembrane apparatus (Gilmour *et al.*, 2003; Lawley *et al.*, 2002a; Lawley *et al.*, 2003a). Each of these proteins are required for plasmid transfer and are predicted to localize to the cell envelope; and all Mpf proteins except TrhN and TrhU are required for production of the conjugative pilus structure (Lawley *et al.*, 2003a). In this chapter, membrane-associated protein complexes minimally comprising the Mpf protein TrhC were visualized as discrete foci

Figure 3-10. Purification and enzymatic analysis of TrhC. Fractions obtained after anion-exchange FPLC were analyzed by SDS-PAGE (lower panel) and an ATPase assay (upper panel). Molecular weight markers are listed on the left. The percent ATP hydrolysis (corrected for background levels of hydrolysis) for each fraction is plotted directly above the respective lane in the coomassie-stained SDS-PAG.



through a TrhC-GFP fusion. The pDT3048 fusion construct was used to address the issues of transfer protein localization and temperature-sensitive DNA transfer. In addition, the assembly requirements for the fluorescent foci were determined by using a library of R27 transfer mutants, and by creating mutations in the TrhC NTP-binding motifs.

Recent findings that conjugative plasmids reside within discrete subcellular positions (Lawley, 2003; Pogliano *et al.*, 2001) raise the possibility that the proteins responsible for plasmid transfer may also be found at similar positions. However, upon examination of *E. coli* carrying pDT3048, there was no tendency for the fluorescent foci representing TrhC-GFP to arrange at the quarter cell or mid cell positions where the self-transmissible plasmids were previously found. Instead, an average of six GFP foci per bacterium were visualized, primarily at the exterior of the cell, with a modest polar distribution. The slight polar distribution of TrhC-GFP may be attributed to the fact that the older pole (i.e. the pole not derived from most recent septation event) is the most static region of the cell in regards to cell wall synthesis, possibly allowing perpetuation of proteins in this region (Lybarger and Maddock, 2001). Furthermore, the relatively unbiased distribution of TrhC-GFP foci within the bacterial membrane suggests that the TrhC transfer protein complex is unlikely to be anchored or has no mechanism to localize to a specific cellular site and is therefore subject to lateral diffusion within the membrane, unlike the replication machinery (Lemon and Grossman, 2000) and plasmids (Lawley, 2003; Pogliano *et al.*, 2001). Likewise, pili produced by the Mpf complexes were also located randomly about the cell. This is consistent with recent observations that the point of fusion between mating pairs of donor and recipient bacteria was located along the lateral wall or at poles of either cell (Lawley *et al.*, 2002b). When expressed in the absence of Mpf proteins, TrhC was found in the cytoplasm and the bacterial cytoplasmic membrane

during cell fractionation experiments. TrhC contains hydrophobic regions that may direct this protein to the bacterial cytoplasmic membrane.

Kumar and colleagues (Kumar *et al.*, 2000) proposed that if a protein were to randomly insert into a membrane then a homogeneous distribution of label would be detected in the membranes, whereas a discrete focus/cluster may represent a specific complex of proteins. In the experimental system used here, GFP foci represent clusters of protein since a lone GFP molecule would be undetectable as a discrete signal. The foci are predicted to represent heterologous complexes of transfer proteins composed partly of TrhC-GFP, rather than homogenous assemblies of TrhC-GFP molecules. The complex nature of a TrhC-GFP focus was revealed by the dependence on twelve other Trh and Tra proteins for assembly. Eleven of the twelve proteins essential for TrhC-GFP foci formation have been classified as Mpf proteins, with the only exception being TraJ. This transfer protein is predicted to have four transmembrane domains by the program TMHMM (Krogh *et al.*, 2001), and is therefore likely to be an inner membrane-associated protein (Figure 2-7). TraJ could possibly interact with other transfer proteins at this cellular location. The protein complex represented as a fluorescent focus may therefore be a subassembly composed of select cell envelope-associated transfer proteins, including both Mpf and non-Mpf proteins.

Distinct protein subcomplexes have also been characterized from the VirB T-DNA transfer apparatus of *A. tumefaciens* that are not composed of the entire complement of VirB proteins, but were proposed to be the core for construction of the remainder of the transfer apparatus (Jakubowski *et al.*, 2003; Krall *et al.*, 2002). These include complexes of VirB6-B8-B9 which can form independently of the other Vir proteins (Jakubowski *et al.*, 2003) and a VirB7-B9-B9-B10 complex that was extracted from the membrane of *A. tumefaciens* (Krall *et al.*, 2002). VirB8 was observed to be essential for assembly of VirB9 and VirB10-associated protein complexes (Kumar *et al.*, 2000). There is no

evidence that the twelve transfer proteins required for formation of TrhC-GFP foci participate in direct interactions with TrhC. Instead, these proteins may cumulatively represent a network of interacting proteins, of which TrhC is a member. The assembly of this network of proteins may be ordered, and loss of an initializing component would likely prevent complete assembly, observed in this study as absence of fluorescent foci. Furthermore, none of TraA, TraK, TraG, and TraI, are required for focus formation, but their absence caused a 25-53% reduction in the number of cells containing foci. These proteins may be part of the interaction network but could play an auxiliary role in complex assembly and are not absolutely required.

The precise functional role of TrhC (and TrhC NTP-binding motifs) in the transfer apparatus is undetermined. These results suggest that the function of the transfer system (including DNA transfer and pilus production) is dependent upon the NTP-binding motifs of TrhC, but that the assembly of the apparatus is not. Various mutations affecting the TrhC NTP-binding domains had little effect on the formation of the TrhC-associated subassembly of transfer proteins. It has been suggested that assembly of VirB protein subcomplexes also do not require NTP binding or metabolism by VirB4 (Dang *et al.*, 1999). VirB4 Walker A motif mutants did not diminish the stimulated uptake of plasmid RSF1010 in recipient cells producing a subset of the VirB proteins, including the VirB4 mutants (Dang *et al.*, 1999). Although NTPase activity has not been demonstrated for TrhC or any related proteins *in vitro*, the *in vivo* conditions may support this energy-providing function for translocation of DNA and/or pilin substrates.

Curiously, the transfer of H-plasmids is inhibited at the temperature in which *S. enterica* sv. Typhi infection occurs. Two scenarios have been hypothesized for the thermosensitive mechanism of IncHI plasmid transfer: (i) essential transfer protein(s) are inactivated at the elevated temperature or (ii) a transcriptional or post-transcriptional mechanism results in temperature-dependent synthesis of essential transfer protein(s)

(Sherburne *et al.*, 2000). Efforts to isolate an R27 mutant that is temperature-resistant for transfer have been unsuccessful, implying that more than one mechanism is present to thermally regulate plasmid transfer. In this study, evidence of distinct mechanisms underlying this phenomenon were demonstrated. There was differential expression of TrhC-GFP from pDT3048 between 27°C and 37°C, and in contrast to results seen at 27°C, the co-existence of pMWG191 and pDT2969 results in a ubiquitous green label throughout the bacterium at 37°C, not distinct foci. This confirms that R27-encoded components are required for foci formation and are not present at the elevated temperature. This data confirms and extends a previous study examining piliation of pDT1942-containing cells at different temperatures (Maher and Taylor, 1993). Using the transmission electron microscope, no pili were observed on cells grown at 37°C, while cells grown exclusively at 27°C exhibited 1-4 pili. The results of Maher and colleagues indicate that temperature-sensitive DNA transfer is due to a lack of pili at 37°C (Maher and Taylor, 1993). The results presented in this chapter suggest that no pili are produced at 37°C because the Mpf proteins necessary for pilus elaboration and DNA transfer are not present at this temperature. It is likely that multiple Tra2 genes are not expressed at 37°C, since a large proportion of Tra2 proteins are believed to be co-translated from a poly-cistronic mRNA (Rooker *et al.*, 1999). It is possible that a protein involved in activating transfer gene expression is temperature labile, as observed for VirA from the Ti plasmid (Jin *et al.*, 1993).

In addition to the decreased TrhC-GFP production at elevated temperatures, a 2.5 hour lag period was required for the visualization of TrhC-GFP after cultures grown at 37°C were cooled to 27°C. Concomitantly, a 3.5 hour lag period was required to observe H-pili after culturing cells at 37°C and then switching to 27°C (Maher *et al.*, 1993). The long lag period seen in our study might represent the time required to reverse conditions that repress Tra2 transfer gene expression, initiate transcription of

transfer genes, translate the resultant mRNA, and then assemble all of the transfer proteins into complexes which can be visualized as discrete fluorescent foci. If TrhC-GFP molecules were visualized immediately after translation (plus the short maturation time of GFPmut2) a much shorter lag time than 2.5 hours would be expected, but it is unlikely that single TrhC-GFP molecules could be detected as discrete foci, thus the foci must represent locations of higher TrhC-GFP density. The long time period required to visualize these foci might primarily consist of the time required to assemble supramolecular complexes. The one hour difference in lag time between the appearance of TrhC-GFP and H-pili is likely due to the period required to assemble the Mpf proteins into a fully functional transfer apparatus and to produce a pilus.

Some cells containing pDT3048 (<1%) still produce GFP foci at 37°C, implying that Tra2 gene repression is not fully comprehensive and a second mechanism to limit plasmid transfer at 37°C must exist. Peter Newnham has shown that plasmid transfer is dramatically and immediately influenced by exposure to 37°C (Newnham, 1995). Since it is unlikely that a transcriptional control mechanism could exert such an effect, it is probable that one or more essential transfer proteins are inactivated or unstable at this temperature. Fullner and Nester (Fullner and Nester, 1996) have shown that while the *vir* genes responsible for T-DNA transfer are expressed maximally at 25°C, the T-DNA transfer apparatus functions with reduced efficiency at temperatures successively greater than 19°C. This can be explained by the observation that the subsistence of VirB10, an essential protein for T-DNA transfer, is temperature-dependent (Banta *et al.*, 1998). These two studies verify that thermosensitivity of T-DNA transfer is a property of the Vir proteins. This model can be extended to R27 where it is possible that one or more of the proteins necessary for H-plasmid transfer is affected by temperature in a manner that prevents conjugation. It is improbable that the pilus is immediately affected

by exposure to 37°C, because H-pilus morphology remained unchanged after a one hour pulse at this temperature (Maher *et al.*, 1993).

Both the intracellular position and temperature-dependent assembly profile of protein complexes whose constituents are essential for the process of bacterial conjugation were identified. These complexes may play a role in pilus production, after which recipient bacteria can be brought towards the donor to form intercellular junctions. The complexes present in these junctions may then participate in DNA transmission between bacteria.

Chapter 4

Interaction between the IncHI1 plasmid R27 coupling protein and type IV secretion system

Portions of this chapter have previously been published as:

Gilmour, M.W., Gunton, J.E., Lawley, T.D., and Taylor, D.E. (2003) Interaction between the IncHI1 plasmid R27 coupling protein and type IV secretion system: TraG associates with the coiled-coil mating pair formation protein TrhB. *Mol Microbiol* **49**: 105-116.

4. Interaction between the IncHI1 plasmid R27 coupling protein and type IV secretion system

4.1 Introduction

During conjugation DNA transfers from one bacterial cell to another; consequently DNA must pass through multiple physical barriers: the cell envelope of both donor and recipient bacteria. This is achieved via the conjugative apparatus, comprised of i) a membrane-associated, multi-protein complex termed the mating pair formation (Mpf) complex responsible for DNA transfer and pilus production, and ii) a cytosolic complex, the relaxosome, which processes plasmid DNA into a single-stranded intermediate for transfer. These two distinct protein complexes are thought to be linked by the inner membrane-associated coupling protein (TraG family), although direct evidence exists only for the relaxosome/coupling protein interaction (Cabezón *et al.*, 1997; Hamilton *et al.*, 2000; Schroder *et al.*, 2002; Szpirer *et al.*, 2000).

The designation of TraG as the R27 coupling protein is based upon several factors, principally the similarity to the model coupling proteins TraD (F), TraG (IncP), TrwB (IncW) and VirD4 (Ti) shown by PSI-BLAST analysis (Lawley *et al.*, 2002a). Additionally, when *traG* is disrupted by transposon insertion, conjugative transfer of R27 ceases entirely, whereas production of H-pili still occurs (Lawley *et al.*, 2002a), implying that TraG is not part of the Mpf complex, but is still required for functional transfer. T-pilus formation on *A. tumefaciens* also occurs in the absence of coupling protein VirD4 (Lai *et al.*, 2000). In Tra1, *traG* is adjacent to *traI* (encoding the R27 relaxase), the characteristic genetic organization of the coupling protein and relaxase in prototypical conjugative systems. The presence of both Walker A and B nucleotide triphosphate binding domains, a hallmark feature of coupling proteins, also correlates with the location of these same motifs in other known coupling proteins (Lessl *et al.*, 1992b; Llosa *et al.*, 1994). These motifs in TraG (IncP) are essential for plasmid RP4 transfer (Balzer

et al., 1994), but NTPase activity was not detected for TraD (F), TraG (IncP), HP0524 (*H. pylori*), and TrwB (IncW) (Moncalian *et al.*, 1999; Schroder *et al.*, 2002).

The goal of this study was to identify Mpf protein(s) that interact with the R27 coupling protein TraG. Using both a bacterial two-hybrid analysis and co-purification of protein complexes, an interaction between TraG and TrhB was detected. The ability of TrhB to form a homomultimer was also demonstrated, allowing a model to be proposed which further defines the conjugative pore and mechanism of conjugative plasmid transfer. In this model, TrhB and TraG form a macromolecular structure that spans the inner membrane and periplasm and may connect with an outer membrane protein to allow passage of plasmid DNA from the donor cell. This study will focus on the characterization of the domain of TrhB which interacts with TraG.

4.2 Experimental Procedures

4.2.1 Bacterial strains and growth conditions

E. coli strains used in this study are presented in Table 4-1. Strains were grown with Luria-Bertani media (Lennox formulation). BTH101 was grown at 30°C and DH5 α at 37°C, unless otherwise noted. Appropriate antibiotics were added to the following final concentrations: ampicillin (Ap) 100 $\mu\text{g ml}^{-1}$; kanamycin (Km) 50 $\mu\text{g ml}^{-1}$; nalidixic acid (Nal) 30 $\mu\text{g ml}^{-1}$; rifampicin (Rif) 20 $\mu\text{g ml}^{-1}$; tetracycline (Tc) 10 $\mu\text{g ml}^{-1}$; and chloramphenicol (Cm) 16 $\mu\text{g ml}^{-1}$. In the BTH assay, X-gal was added to a final concentration of 40 $\mu\text{g ml}^{-1}$ in agar plates containing Amp and Km, and colonies from each pairwise combination were restreaked then inoculated into 20 ml LB and grown for 16 hours at 28°C for subsequent quantification of β -galactosidase activity in a standard Miller assay (Miller, 1972). Complementation experiments were performed as described (Lawley *et al.*, 2002a; Taylor and Levine, 1980) between donor cells (RG192 + drR27

Table 4-1. Plasmids and *E. coli* strains used in this chapter.

Strain or Plasmid	Description	Selective markers ^a	Source
<i>E. coli</i> strain			
BTH101	F ⁺ <i>cya</i> -99 <i>ara</i> D139 <i>gal</i> E15 <i>gal</i> K16 <i>rps</i> L1 (Str ^r) <i>hsd</i> R2 <i>mcr</i> A1 <i>mcr</i> B1	Str	Karimova <i>et al.</i> , 1998
DH5 α	λ ϕ 80d/ <i>lac</i> Z Δ M15 Δ (<i>lac</i> ZYA- <i>arg</i> F)U169 <i>rec</i> A <i>end</i> A1 <i>hsd</i> R17(<i>r_k</i> ⁺)	Invitrogen	
DH10B	F ⁺ <i>mrc</i> A Δ (<i>mrr</i> - <i>hsd</i> RMA- <i>mrc</i> BC) ϕ 80d/ <i>lac</i> Z Δ M15 Δ <i>lac</i> X74 <i>deo</i> R <i>rec</i> A1 <i>end</i> A1 <i>ara</i> D139 Δ (<i>ara</i> , <i>leu</i>) 7697 <i>gal</i> U <i>gal</i> K λ - <i>rps</i> L <i>hup</i> G	Invitrogen	
DY330N	W3110 Δ <i>lac</i> U169 <i>gal</i> 490 λ c1857 Δ (<i>cro</i> - <i>bio</i> A); ⁺ spontaneous Nal ^r	Nal	Lawley <i>et al.</i> , 2002a
RG192	<i>ara</i> <i>leu</i> <i>lac</i>	Rif	Taylor, 1983
Plasmids			
pKT25	p15A origin of replication; encodes CyaA ₁₋₂₂₄	Km	Karimova <i>et al.</i> , 1998
pUT18C	ColE1 origin of replication; encodes CyaA ₂₂₅₋₃₉₉	Ap	Karimova <i>et al.</i> , 1998
pKT25- <i>zip</i>	leucine zipper of GCN1 (BTH positive control)	Km	Karimova <i>et al.</i> , 1998
pUT18C- <i>zip</i>	leucine zipper of GCN1 (BTH positive control)	Ap	Karimova <i>et al.</i> , 1998
pKT25- <i>tra</i> G	2.1kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pKT25	Km	Gilmour <i>et al.</i> , 2003
pUT18C- <i>tra</i> G	2.1kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pUT18C	Ap	Gilmour <i>et al.</i> , 2003
pKT25- <i>trh</i> B	1.4kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pKT25	Km	Gilmour <i>et al.</i> , 2003
pUT18C- <i>trh</i> B	1.4kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pUT18C	Ap	Gilmour <i>et al.</i> , 2003
pUT18C- <i>trh</i> B ₁₋₂₂₀	0.7kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pUT18C	Ap	Gilmour <i>et al.</i> , 2003
pUT18C- <i>trh</i> B ₁₋₁₃₃	0.4kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pUT18C	Ap	Gilmour <i>et al.</i> , 2003
pUT18C- <i>trh</i> B ₂₀₄₋₄₅₂	0.8kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pUT18C	Ap	Gilmour <i>et al.</i> , 2003
pKT25- <i>tra</i> H	0.5kbp <i>Bam</i> HI/ <i>Kpn</i> I-digested PCR product in pKT25	Km	Gilmour <i>et al.</i> , 2003
pMS119EH	Cloning vector; <i>P_{lac}</i> - <i>lac</i> F ⁺ ; pMB1 origin of replication	Ap	Strack <i>et al.</i> , 1992
pMS119EH- <i>trh</i> B _{FLAG}	1.4kbp <i>Eco</i> RI/ <i>Bam</i> HI-digested PCR product in pMS119EH	Ap	Gilmour <i>et al.</i> , 2003
pTL42	<i>Trh</i> B _{H168} expression vector (pMS119EH)	Ap	Lawley <i>et al.</i> , 2003a
pTL43	<i>Trh</i> B expression vector: 1.4kbp <i>Eco</i> RI/ <i>Bam</i> HI-digested PCR product in pMS119EH	Ap	Gilmour <i>et al.</i> , 2003
pDT1942	derepressed transfer mutant of R27	Tc, Km	Maher <i>et al.</i> , 1991
pDT2971	pDT1942 with mini::Tn10 inserted into <i>trh</i> B	Tc, Km, Cm	Rooker <i>et al.</i> , 1999
pDT2989	pDT1942 with mini::Tn10 inserted into <i>tra</i> G	Tc, Km, Cm	Lawley <i>et al.</i> , 2002a

a. Antibiotic resistances: Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Cm, chloramphenicol; Nal, nalidixic acid; Str, streptomycin

transfer mutant + complementation vector) and recipient cells (DY330N). Both donor and recipient strains were grown at 28°C.

4.2.2 DNA manipulations

R27 transfer genes and truncated fragments were cloned into the BTH vectors after PCR amplification with *Taq* polymerase (Invitrogen) and primers listed in Table 4-2 and digestion with restriction enzymes *Bam*HI and *Kpn*I (Invitrogen). After ligation, the products were transformed into DH5 α and screened for productive cloning by plasmid isolation and restriction analysis. Cloning into pMS119EH was with *Eco*RI and *Bam*HI (Invitrogen).

4.2.3 Immunoprecipitation of TrhB/B and TrhB/TraG complexes

Following the procedures of Tang *et al.* (Tang *et al.*, 1996) and Ho *et al.* (Ho *et al.*, 2002), 1 ml of overnight culture was used to inoculate 20 ml LB + appropriate antibiotics, grown to mid-log at 30°C, and then induced with 0.4 mM IPTG for one hour. Bacteria were harvested and resuspended in 0.5-1 ml of IP buffer (PBS pH 7.4, 0.1% Triton X-100, 100 mM NaCl, 5 mM EDTA and 10% glycerol) + 0.5 mM PMSF; then 10 μ g lysozyme was added and the mixture left on ice for 30 minutes. Cells were further lysed by sonication (3 minutes with intermittent 10 second breaks, Fisher model 300 sonicator). Cell debris was cleared by centrifugation at 16 000 rpm for 15 minutes, then 40 μ L of ANTI-FLAG M2-agarose affinity gel (Sigma) in a 1:1 slurry with IP buffer was added to the supernatant and rotated at 4°C for 1.5 hours. The affinity gel was then washed three times with 1 ml of IP buffer and once with 1 ml IP buffer + 0.5 mg/ml Angiotensin I (Sigma). Proteins were eluted from the gel by boiling in LSB buffer + DTT and run on a 10% SDS-PAGE, transferred to nitrocellulose, and blocked with 10% (w/v) milk, 0.1% Tween 20 in PBS, pH 7.4 (MPBST). Primary antibody was applied [anti-

Table 4-2. Primers used to clone *trhB*, *traG* and *traH* (and derivatives) into BTH and expression vectors

Primer	Sequence (5' to 3') ^a
<i>traG</i> (BTH-forward)	TATAGGATCC <u>G</u> ACAAAATCAAAAAGAACCAAC
<i>traG</i> (BTH-reverse)	AAGGTACCC <u>CA</u> TGCAATTTCCTTAGATATTTA
<i>trhB</i> (BTH-forward)	TATAGGATCC <u>AG</u> ACATTAAAAAGGCCTGGGAAAATAAAAC
<i>trhB</i> (BTH-reverse)	TATAGGTACCC <u>GC</u> GCGGCTTGTCAGGTTTGTAG
<i>trhB</i> ₁₋₂₂₀ (BTH-reverse)	TATAGGTACCC <u>AT</u> GCGAATAGTTCGCGTTGGGGCCTTTTC
<i>trhB</i> ₁₋₁₃₃ (BTH-reverse)	TATAGGTACCC <u>AT</u> GCCTGCATCGAGATTGCGATTAC
<i>trhB</i> ₂₀₄₋₄₅₂ (BTH-forward)	TATAGGATCC <u>AG</u> GGCGCGAGGTTGTTGCGGGAAGTG
<i>trhB</i> (EH-forward)	TATAGAATTCAATGGACATTAAAAAGGCCTG
<i>trhB</i> _{FLAG} (EH-reverse)	ATATGGATCC <u>TTA</u> CTTGTCATCGTCGTCCTTGTAGTCGCGGCTTGTCAGGTTTGTAG
<i>trhB</i> (EH-reverse)	TATAGGATCC <u>CT</u> CAGCGGCTTGTCAGGTTTG
<i>traH</i> (BTH-forward)	TATAGGATCC <u>GT</u> CTAAATCAAGCTATTAAAG
<i>traH</i> (BTH-reverse)	TATAGGTACCTCATTGTGCCACCTCTGATTTTG

a. Restriction sites are underlined; bold letters indicated engineered bases to maintain a correct reading frame or an engineered stop codon

adenylate cyclase rabbit serum, #L24023, specific for the catalytic domain (D. Ladant)], washed, then secondary antibody (anti-rabbit HRP, Sigma) was applied for one hour. Detection was with SuperSignal West Dura extended substrate (Pierce).

4.2.4 Solubilization/purification of TrhB_{HIS6} and far-Western analysis

A two litre culture of *E. coli* DH10B containing pTL42 was grown to mid-log at 37°C/200 rpm then induced with 0.4 mM IPTG for one hour at 37°C/200 rpm. Cells were harvested by centrifugation, washed in buffer A (10 mM Tris-HCl pH 7.6 and 5 mM MgCl₂), resuspended in 15 ml of buffer A, and then lysed by three passages through a French press (8000 psi). Unlysed cells were removed by two low-speed centrifugations and the membrane fraction collected by ultracentrifugation (Ti70.1 rotor, 35 000 rpm, 4°C, 30 min.) The pellet was washed with buffer A, then resuspended in 10 ml buffer B (50 mM Tris-HCl pH 7.6, 500 mM NaCl, 2% Triton X-100, 10% glycerol) + 10 mM imidazole by gently rotating for 3 hours at 4°C. To clear insoluble material, another low-speed spin was performed (JA25.15 rotor, 8000 rpm, 4°C, 10 min.). The supernatant was applied overnight at 4°C to 2 ml Ni-NTA Superflow resin (Qiagen). Unbound material was removed with three 12 ml washes in buffer B + 10 mM imidazole, and TrhB was eluted with an imidazole gradient of 50-300 mM in buffer B. The eluted protein was confirmed as TrhB by tandem mass spectrometry. TrhB primarily eluted with 200 mM imidazole at ~50 µg/ml.

For far-Western analysis of TrhB/TrhB interactions, protein samples were prepared from cultures containing various expression constructs grown at 28°C/200 rpm until mid-log phase (OD₆₀₀ ~ 0.6), and induced with 0.4 mM IPTG at 28°C/200 rpm for one hour. Cells from 1 ml of culture were harvested by centrifugation and boiled for 5 minutes in 50 µl LSB + DTT, and 15 µL were loaded onto a 10% SDS-PAGE. Proteins were visualized by Coomassie stain or transferred to nitrocellulose, blocked for 1 hour with 8% MPBST,

and incubated with 1 ml soluble TrhB_{His6} in buffer B + 200 mM imidazole and diluted in 9 ml 5% MPBST overnight at 4°C. Unbound protein was removed by washing 3x10 minutes with 5% MBST, and bound TrhB_{His6} detected by immunoblotting with 100 ng/ml monoclonal Penta-His antibody (Qiagen).

4.2.5 Membrane preparations

500 ml of LB-Lennox with ampicillin was inoculated with 20 ml of an overnight culture of DH10B (pTL42), grown at 28°C/200 rpm until mid-log phase ($OD_{600} \sim 0.8$), and induced with 0.4 mM IPTG at 28°C/200 rpm for one hour. Cells were harvested by centrifugation (10 min., 6000 rpm, 4°C) and resuspended in 10 ml of ice-cold buffer A with 1 mg of RNase and 100 U of micrococcal nuclease. Cells were lysed by three passages through a French pressure cell at 8000 psi and 1 mg of lysozyme added for 30 minutes on ice. Unlysed cells were removed by centrifugation (2 times; 7 min., 6000 rpm, 4°C), and the supernatant subjected to ultracentrifugation (2 hours, 35000 rpm, Ti70.1 rotor, 4°C) to isolate soluble (cytoplasmic/periplasmic) and insoluble (membrane) fractions. The membrane fraction was resuspended in 55% sucrose (w/w), 10 mM Tris-HCl pH 7.6, 5 mM EDTA for further analysis.

4.2.6 Production of antisera to TrhB and detection of TrhB in cell lysates

A peptide representing the C-terminus of TrhB (CTCqkgatipnltsr) was synthesized and conjugated to carrier protein KLH (via the above capitalized amino acids) and rabbits were immunized with 200 µg of peptide in complete Freund's adjuvant at week 0 (ProSci Incorporated). Subsequent immunizations at weeks 3, 6 and 10 were with 100 µg of peptide in incomplete Freund's adjuvant. Serum was collected at week 13.

For detection of TrhB in whole-cell lysates, overnight cultures of RG192 + drR27 and derivatives were grown at 28°C. The optical density of each culture was determined at 600 nm, then the cells were harvested and resuspended in appropriate volumes of PBS (pH 7.4), 0.5% Triton X-100, 10 mM EDTA to equalize cell density (~1 ml). Samples were prepared by boiling in 2x LSB sample buffer with DTT, and 5 µL loaded onto a 10% acrylamide gel. The immunoblot was processed as above, except a 1:2000 dilution of TrhB antisera was used as the primary antibody.

4.2.7 Web-based computer programs

Coils (www.ch.embnet.org/software/COILS_form.html); PSI-BLAST (www.ncbi.nlm.nih.gov/BLAST/); TMHMM (www.cbs.dtu.dk/services/TMHMM-2.0/); and EMBOSS pairwise local alignment (Blosum40 matrix, gap penalty =10, extension penalty =0.5; www.ebi.ac.uk/emboss/align/) were used.

4.3 Results

4.3.1 In vivo detection of TraG and TrhB interactions

A bacterial two-hybrid (BTH) system was employed to characterize the membrane-associated complex of R27 transfer proteins. In this BTH assay, test proteins are fused to one of two adenylate cyclase fragments (AC18 or AC25), and upon interaction of the two investigated proteins within a cyclase-deficient strain of *E. coli*, the cyclase fragments are brought into close proximity leading to the conversion of ATP to cAMP (Karimova *et al.*, 1998). This molecule is a positive activator of catabolic operons, including the *lac* operon, and induction of β -galactosidase enables chromogenic differentiation between interacting and non-interacting clones. This BTH system has been used successfully to study the interactions between other conjugative transfer

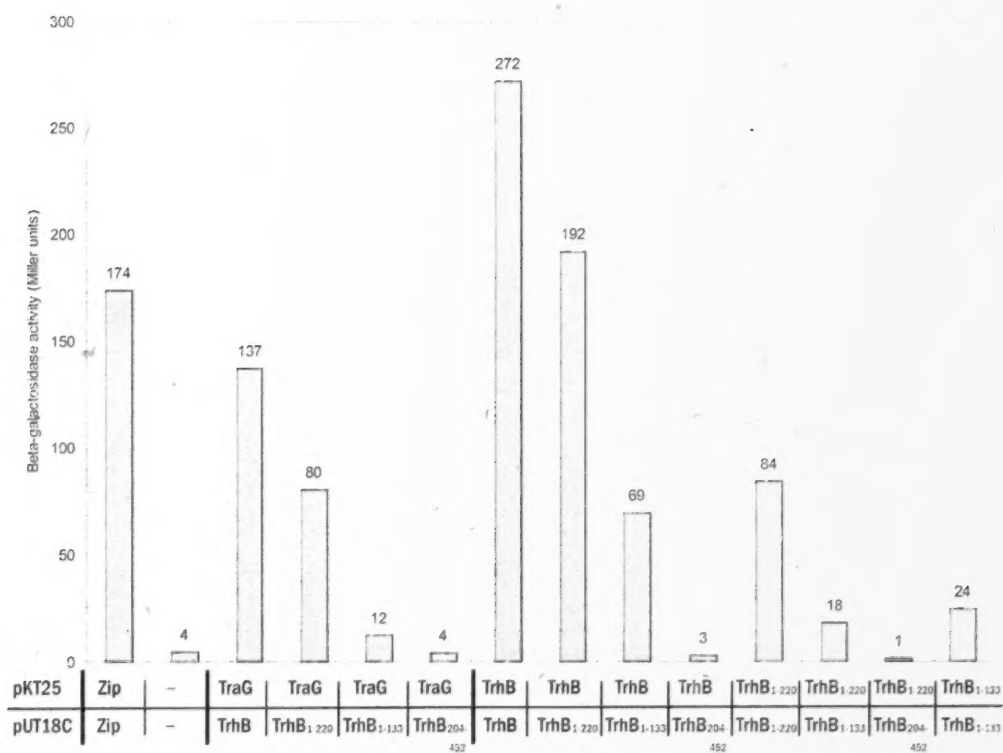
proteins (Szpirer *et al.*, 2000) and is appropriate for the study of multi-component protein complexes and membrane-associated protein complexes (Karimova *et al.*, 1998).

To identify Mpf protein(s) that interact with TraG, the R27 Mpf genes (*trhB*, -E, -L, -C, and -V) were cloned into the bacterial two-hybrid vector pUT18C and co-transformed them with pKT25-*traG* into *E. coli* BTH101 (Table 4-1 and Table 4-2). Each of these fusion clones places a CyaA subunit, AC18 or AC25, on the N-terminus of the transfer protein. Only one productive pairwise combination was observed: TraG_{AC25} with TrhB_{AC18}. Within 40 hours after plating, >99% of the BTH101 + pKT25-*traG* + pUT18C-*trhB* colonies turned blue, whereas the BTH positive control (leucine zipper) was also blue, and the negative control (empty BTH vectors) colonies remained white or had faint blue centers (data not shown). Quantification of β -galactosidase activity was performed using a standard Miller assay (Figure 4-1). These data indicate that TraG interacts *in vivo* with TrhB. To my knowledge, this is the first demonstration of a molecular interaction between the coupling protein and an Mpf component.

To determine the domain of TrhB which interacts with TraG, deletion constructs of *trhB* were created (Figure 4-2), cloned into pUT18C, and co-transformed with pKT25-*traG*. Sites for N- and C-terminal truncations of TrhB were selected by comparison with TrhB homologues, and the point of truncation placed in less-conserved regions (discussed below). The three constructs include N-terminal fusions of AC18 to the first 133 aa, the first 220 aa, and the last 249 aa of TrhB (TrhB₁₋₁₃₃, TrhB₁₋₂₂₀, and TrhB₂₀₄₋₄₅₂, respectively). After growth on indicator plates, each pairwise TraG/TrhB combination was tested for β -galactosidase activity (Figure 4-1). Only the TrhB₁₋₂₂₀ peptide fusion construct had a significant level of activity with TraG_{AC25} above the negative control values, whereas TrhB₁₋₁₃₃ or TrhB₂₀₄₋₄₅₂ co-produced with TraG_{AC25} resulted in low levels

Figure 4-1. Bacterial two-hybrid analysis of TraG-TrhB and TrhB-TrhB interactions.

Full-length and truncated TrhB fusion constructs were co-produced with either TraG or TrhB cyclase fusion constructs in BTH101 and liquid cultures were analyzed for β -galactosidase activity. A representative experiment is shown.



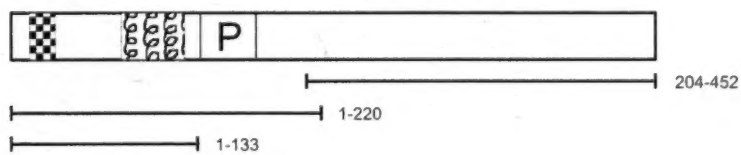
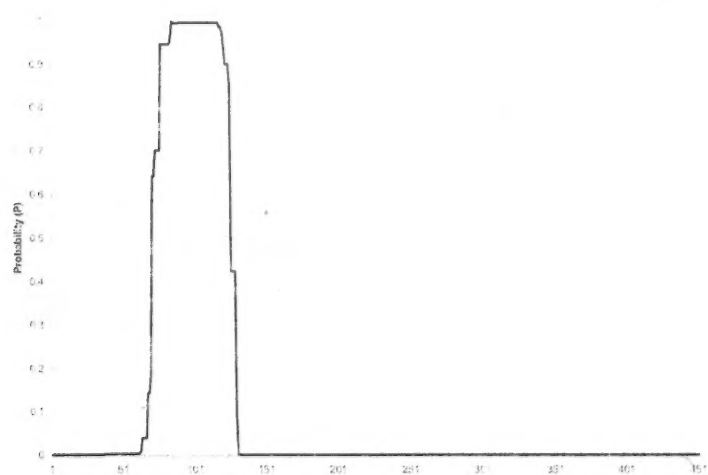
of cyclase activation. These data indicate that TraG associates with the N-terminal half of TrhB, but not the C-terminus.

Using the Coils program (Lupas *et al.*, 1991), TrhB was predicted to contain a coiled-coil region from residues 77-124 (Figure 4-2). This domain could aid in the oligomerization of TrhB into a higher order complex, therefore the TrhB and TrhB deletion constructs were tested in the BTH assay (Figure 4-1). A positive interaction was found when full-length TrhB was co-transformed with TrhB, TrhB₁₋₂₂₀, and TrhB₁₋₁₃₃, but not with TrhB₂₀₄₋₄₅₂, indicating that the N-terminus of TrhB acts as the homotypic interaction domain. The positive interaction between TrhB/TrhB₁₋₁₃₃ correlates with the predicted coiled-coil domain. Co-expression of the N-terminal regions TrhB₁₋₂₂₀/ TrhB₁₋₂₂₀ also produced a positive BTH signal, however TrhB₁₋₂₂₀/ TrhB₁₋₁₃₃ and TrhB₁₋₁₃₃/ TrhB₁₋₁₃₃ were much less productive. This may be due to an additive effect elicited by the truncated peptides which, individually, have decreased interaction abilities. Using the two slightly overlapping TrhB N- and C-terminal halves TrhB₁₋₂₂₀ and TrhB₂₀₄₋₄₅₂, no positive BTH response was detected, and it is therefore unlikely that there is re-association between these segments of TrhB. To confirm that TrhB truncations are stably expressed, and that non-productive BTH responses did not result from a lack of protein production, TrhB₂₀₄₋₄₅₂ was probed for and detected using an antibody specific for the last 13 amino acids of TrhB (Table 4-3). Although this antibody does not recognize N-terminal segments of TrhB, both TrhB₁₋₂₂₀ and TrhB₁₋₁₃₃ were both productive in the BTH assay (Figure 4-1 and Table 4-3), implying that they are produced. It cannot be ruled out that decreased levels of protein production resulted in a decreased BTH response.

A functional analysis of transfer protein/adenylate cyclase fusion constructs by using the BTH constructs in a complementation assay of transfer mutants was performed. The BTH vectors pUT18C-*traG* and pUT18C-*trhB* were transformed into RG192 cells

Figure 4-2. Schematic representation of TrhB and TraG. Predicted transmembrane regions are illustrated as checkered boxes, coiled-coil regions by boxes with spirals, proline-rich domains by a boxed letter P and nucleotide triphosphate-binding domains (Walker A and B) with solid black boxes. Horizontal lines show TrhB truncation constructs used for BTH analysis.

TrhB



TraG



Table 4-3. Properties of TrhB and TrhB derivatives produced from pUT18C.

<i>trhB</i> -pUT18C clone	Bacterial two-hybrid response ^a		Protein production ^b	Domains present ^c
	<i>traG</i> -pKT25	<i>trhB</i> -pKT25		
1-452	+	+	+	CC/Pro
1-220	+	+	N/A	CC/Pro
1-133	-	+	N/A	CC
204-452	-	-	+	-

a. Adenylate cyclase activity in BTH101 containing pKT25 and pUT18C vectors

b. As determined by immunoblot analysis of induced whole cell lysates using anti-TrhB antibody specific for C-terminus (N/A = not applicable)

c. CC = coiled-coil; Pro = proline rich domain (see text)

containing the respective R27 transfer mutant and mated with DY330N (Table 4-4). Although full complementation was not achieved with any fusion construct, transfer ability was partially restored with full-length TraG and TrhB constructs, and therefore each was able to support conjugative transfer. TrhB peptides 1-220 or 204-452 were unable to support conjugative transfer, likely due to the loss of required functional domains.

4.3.2 Immunoprecipitation of transfer protein complexes

To confirm the *in vivo* interaction between TraG and TrhB and the homotypic interaction of TrhB, protein complexes were purified from DH5 α cells producing epitope-tagged transfer proteins (Figure 4-3). TrhB with a C-terminal FLAG tag (TrhB_{FLAG}) and cyclase fusion constructs of either TraG or TrhB was used. The TrhB_{FLAG} construct, which produces TrhB with a C-terminal Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys tag, was able to fully complement a *trhB*⁻ mutant of R27 (Table 4-4). TrhB_{AC25} and TraG_{AC25} were each co-precipitated by TrhB_{FLAG}, indicating that both TrhB and TraG interact with TrhB. As a negative control, TrhB_{FLAG} was omitted to ensure that the fusion constructs were not associating with components of the immunoprecipitation reaction (Figure 4-3A). In these reactions, no TrhB or TraG protein was detected with the anti-adenylate cyclase antibody. A protein species of higher molecular weight than TrhB_{AC25} appeared in Coomassie and Sypro Ruby stains of all immunoprecipitation reactions (data not shown), and was detected by the anti-adenylate cyclase antibody in immunoblots (Figure 4-3A, TrhB_{AC25} reactions). The identity of this ~85 kDa species was unknown, however, TrhB_{AC25} was resolved from this species to allow differentiation and identification of TrhB_{AC25}-containing precipitations.

To verify that positive associations were not a result of protein over-expression or non-specific association between the AC25 subunit and TrhB_{FLAG}, an

Table 4-4. Functional analysis of TrhB and TraG fusion constructs.

Complementation experiments were performed with donor cells containing expression vectors and an appropriate R27 transfer mutant.

Complementation Vector	Relative Transfer Frequency ^a
pMS119EH- <i>trhB</i> _{FLAG} ^b	4.4
pUT18C- <i>traG</i> ^c	6.1×10^{-4}
pUT18C- <i>trhB</i> ^b	5.1×10^{-4}
pUT18C- <i>trhB</i> ₁₋₂₂₀ ^b	0
pUT18C- <i>trhB</i> ₂₀₄₋₄₅₂ ^b	0

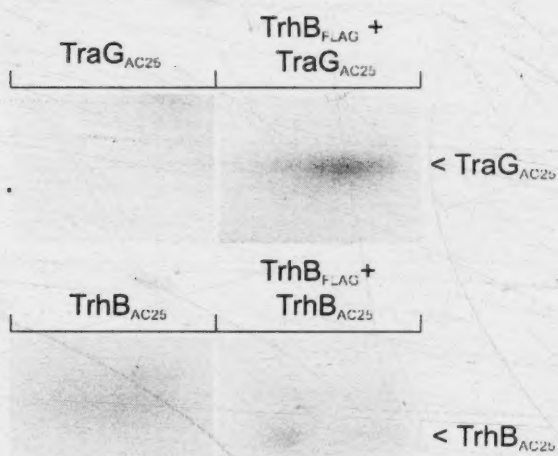
a: in comparison to transfer frequency of pDT1942 (measured as transconjugants/donor), and where an equal transfer frequency during complementation would be a relative transfer frequency =1

b: complementation of pDT2971

c: complementation of pDT2989

Figure 4-3. Co-immunoprecipitation of TrhB-TraG and TrhB-TrhB complexes. (A) Cell lysates containing the listed proteins were incubated with M2 anti-FLAG affinity gel, washed, and resolved by SDS-PAGE. After transfer to nitrocellulose, the precipitated proteins were probed with anti-adenylate cyclase antibody. (B) TraH_{AC25} and TrhB_{FLAG} were produced in DH5 α , lysed and insoluble material removed by centrifugation. This 'cleared lysate' was incubated with M2 anti-FLAG affinity gel and processed as above (lane marked 'Immunoprecipitation').

A.



α -adenylate cyclase

B. TrhB_{FLAG} + TraH_{AC25}

Cleared Lysate	Immuno- precipitation

< TraH_{AC25}

α -adenylate cyclase

immunoprecipitation assay with TraH_{AC25} and TrhB_{FLAG} was performed (Figure 4-3B). TraH is encoded in the Tra1 region of R27 and has been proposed to be a relaxosomal protein (Lawley *et al.*, 2002a). There is no previous evidence of an interaction between this 19.2 kDa transfer protein and TrhB, and these proteins were negative in a BTH assay (data not shown). TraH_{AC25} was stably expressed and was soluble under the same conditions used for the TrhB and TraG assays (Figure 4-3B, cleared lysate), yet was not detected in immunoprecipitated material (Figure 4-3B, immunoprecipitation). These data support our observations of TraG-TrhB and TrhB-TrhB protein interactions.

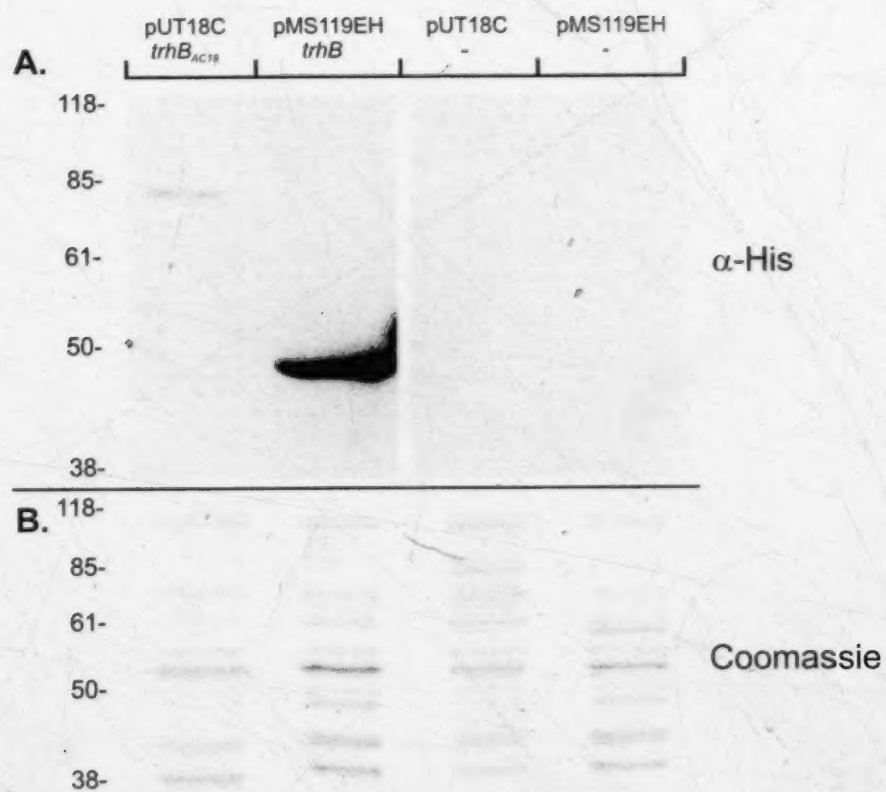
4.3.3 Far-Western Analysis of TrhB interactions

The TrhB-TrhB protein-protein interaction was further confirmed by far-Western analysis. Cell lysates containing native TrhB (predicted molecular weight of 48 kDa) produced from pMS119EH, or TrhB_{AC18} (predicted molecular weight of 66 kDa) produced from pUT18C, and lysates of cells with pMS119EH or pUT18C were run on a SDS-PAGE, transferred to nitrocellulose, and probed with soluble TrhB_{His6}. After washing unbound TrhB_{His6}, the remaining TrhB_{His6} was detected by Western analysis (Figure 4-4A). It was found that TrhB_{His6} interacts with proteins with molecular weights corresponding to TrhB_{AC18} and TrhB. Each of these proteins were minor species within the probed cell lysate (Figure 4-4B), indicating that TrhB_{His6} interaction was not resulting from non-specific interactions to a predominant species. TraG/TrhB interactions could not be detected using this method, possibly because due to the denaturing conditions during gel electrophoresis.

4.3.4 Stability of TrhB in a *traG* mutant

It is common that the stability of a protein is dependent upon the presence of the interacting partner(s). TrhB was probed for in lysates of *E. coli* cells containing either

Figure 4-4. Far-Western detection of TrhB-TrhB interactions. Cell lysates containing various expression constructs were resolved by 10% SDS-PAGE then (A) immobilized on nitrocellulose, probed with soluble TrhB_{His6} and interactions detected by immunoblotting with Penta-His antibody, or (B) stained with Coomassie blue R-250. Approximate molecular weights (kDa) are listed.



derepressed R27 (drR27), a *trhB* transposon mutant of drR27 (with and without the TrhB_{FLAG} expression plasmid) or a *traG* transposon mutant of drR27 using polyclonal antiserum raised against a peptide representing the last 13 amino acids of TrhB. After immunoblotting, TrhB was detected in the lysates from drR27 and *traG* R27 mutant-containing cells, but not for the *trhB* R27 mutant (Figure 4-5). Therefore, the presence of TraG does not influence the production/stability of TrhB. This result is supported by an Hgal phage binding assay, wherein the above *traG* R27 mutant was still capable of producing H-pili (Lawley *et al.*, 2002a). Hence, the Mpf apparatus, presumably including TrhB, is still functional in the absence of TraG. TrhB_{FLAG} production was also identified, and there was a detectable difference between in the apparent molecular weight of this epitope-tagged construct and native TrhB (Figure 4-5).

4.3.5 Membrane localization of TrhB

Computational analysis of the TrhB primary amino acid sequence with TMHMM predicted a transmembrane (TM) region at residues 12-34 (Figure 4-6). Subcellular fractionation studies were performed to confirm the predicted membrane localization of TrhB. After cell lysis, ultracentrifugation was used to isolate soluble (cytoplasmic and periplasmic) and insoluble (membrane) fractions. TrhB was found exclusively in the particulate fraction (data not shown), suggesting membrane localization. Attempts to determine the localization of TrhB to a specific membrane fraction using flotation, isopycnic and continuous sucrose gradients or buffered sucrose cushions were unsuccessful (data not shown). To recover TrhB from soluble fractions of cell lysates during TrhB_{His6} purification and TrhB_{FLAG} immunoprecipitation reactions detergent was required at all steps (Figures 4-3 and 4-4), further implying that TrhB is membrane-associated.

Figure 4-5. Production and stability of TrhB. Immunoblot of whole-cell lysates of RG192 containing drR27, or drR27 transfer mutants of *traG* or *trhB*, or *trhB* drR27 transfer mutant with pMS119EH-*trhB*_{FLAG} (no IPTG induction) probed for TrhB content with TrhB-specific antisera. Approximate molecular weights (kDa) are listed on the left and approximate location of TrhC and TrhB_{FLAG} on the right.

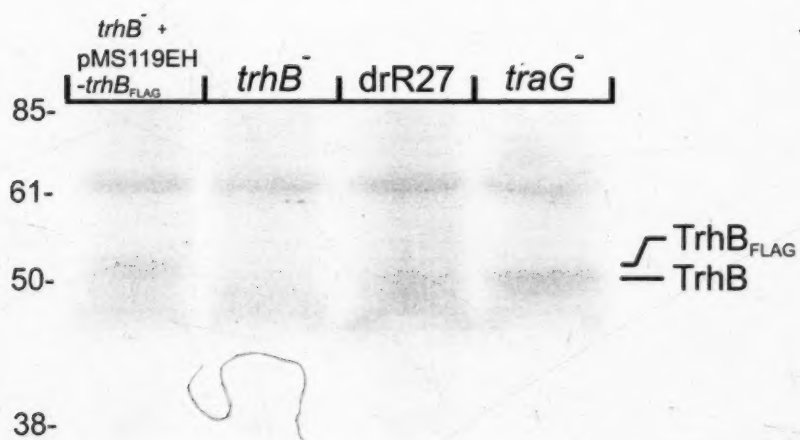


Figure 4-6. Multiple sequence alignment and primary/secondary sequence analysis of TrhB and homologues. The alignment was generated using ClustalW in MegAlign (DNASStar) and conserved residues were shaded using GENEDOC. Underlined residues indicate transmembrane helices predicted by TMHMM, red coloured residues indicated coiled-coil domains predicted by Coils, and all proline residues are coloured green.

Accession numbers: R478 (CAE51539), R27 (NP_058242), Rts1 (NP_640170), pCAR1 (NP_758672), SXT (AAL59682), R391 (AAM08009), pRS241d (ZP_00008313), pSLT (NP_490567), R100 (NP_052951), pNL1 (NP_049165), F (AAC44179).


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TrhB R478 : -----MDIKKAWENKTVRIS---VIGALLVVIYISQSIFSTVKKKKTKKKD : 47
TrhB R27 : -----MDIKKAWENKTVRIS---VIGAALMVIVISQSIFSTVKKKKTKKKD : 47
210 Rts1 : -----MIFMEKIKDLWANLDAKKRYALWA LAIVITLIM-PENSSRKATREK : 53
TrhB pCAR1 : -----MKAIQOFLEKLT RGKLMIAIGTVLIGTVGAMNAAT ERKRAAAK : 51
TrhB SXT : -----MKAQWEQMSN KRGLSVA IAGGLILMFS-PNDDGS SRNRQE : 47
TrhB R391 : -----MKAQWEQMSN KRGLSVA IAGGLILMFS-PNDDGS SRNRQE : 47
4262 pRS241d : -----MSSDARKKQQM VLGLIGGLLLIIVLGG MNRKQAAFRS GA DRV : 48
TrhB pSLT : -----MASINTVKKRQY WLGI VAV AAAAGGGY SDVMSGNG -AAEQE : 48
TrhB R100 : -----MASINTVKKRQY WLGI VAV TASAAGGA Y SDVMSGNGE -VAEQE : 49
TrhB pNL1 : MDWKN FRKG KALDGAATH GSGDAS LGDAIVANEAVRKQMLL--AG LAAL GSSW FAGDHKSSDA -VKROV : 78
TrhB F : -----MASINTVKKRQY WLGI VAV TASAAGGA Y SDVMSGNGE -VAEQE : 49

TrhB R478 : MQTNLLDDSQMNKLSNEESQKVYKEMVKQNRDQNAAKED-----KRAEKAQNETKAQVASLTSQLQLSQQIND : 119
TrhB R27 : MQTGFLDDSQMNKLSNEESQKTYNEMVRQNRDQNAAKAD-----KDAEKAQENKAQIASLASQVQLTTLTE : 119
210 Rts1 : VIGNLTDSTTGVSQNAIAARLRS DETIKSTKTAVDTIN-----KTIDPDALNK--RLTNENVQKNZAAARQT : 122
TrhB pCAR1 : QVEALTDDETRALGMSIASOLRQQAQONFBERINGQKE-----KDVQLKNTQE---KVSGAEQELQLVQKELAK : 120
TrhB SXT : TIRHLTDTNTRDVGIDSLAANVKLSERNEQPREVERLR-----KDVDSGRLSF---GSPTSSEVNALAR--- : 113
TrhB R391 : TIRHLTDTNTRDVGIDSLAANVKLSERNEQPREVERLR-----KDVDSGRLSF---GSPTSSEVNALAR--- : 113
4262 pRS241d : DETIADRTSNAS FLSWARQSAEQDQLNKTTELTNTVVK-----GMAEKQASDLRE-----LRTQYDEVIVQ--- : 112
TrhB pSLT : EVDT TGVVDTTFDDKVR-QHATTEQVTAACQKQYDEIR-----HELDVLNKQRC-----DDURRIEK--- : 107
TrhB R100 : EVDT TGVVDTTFDDKVR-QHATTEQVTAACQKQYDEIR-----HELDVLNKQRC-----DDURRIEK--- : 108
TrhB pNL1 : ADVS DEMVMKNMAEKWRASQSEACMSMDTNRALAARAQRADQLEAQLAQGQAQSTSG---GGSPDTERVLSA--- : 152
TrhB F : EVDT TGVVDTTFDDKVR-QHATTEQVTAACQKQYDEIR-----HELDVLNKQRC-----DDURRIEK--- : 108

TrhB R478 : OTNRNGNANLDAGSSRKTINEQA AA PYQLNANA INGVN NYASIT TRNS MRTITQSSIKINGTDGVIQVM VSENR : 200
TrhB R27 : OTSRNGNRNLDAGGPRNNVNEQA PAKPYQLNANAANGVSSGYA ISPTRNS MRTITQSSIKINGTDGVIQVM VSENR : 200
210 Rts1 : UNRIDTHIKN PASSTGGA-----LNKNVFAA QDQSGEAAENQONARATASGTLSVYENDIA DSDPDHADA : 197
TrhB pCAR1 : GREKKGTGESEDNSSDS-----SASNOARS IP-----RESVFVNKATSS AELTDVYNSEEQ---ED KSR : 184
TrhB SXT : RAELDDVRAGGDSVVEG-----TNSRFEV LS-----AMELKDEK LFS-NDDYFANAL DLYQVANGQ : 177
TrhB R391 : RAELDDVRAGGDAAVEG-----TNSRFEV LS-----AMELKDEK LFS-NDDYFANAL DLYQVANGQ : 177
4262 pRS241d : QQAEINSIKTGA VAVG-----GETGEAQA G-----YGQFVSGGTRTAAGRAGQGGAAG--GA GAA MV : 175
TrhB pSLT : GGDNAALAEQVKALGAN-----VTTIGE V-----HTVSPFGEGETQ GNTMTTFPQG--AVPPTAFY : 170
TrhB R100 : GGDNAALAEQVKALGAN-----VTATGE V-----QMPAPFGEGETQ GNTVSPFQGSVAVPPTAFY : 173
TrhB pNL1 : YQENENQKALAAARQS-----VMGAGAT V-----VGNALYGRIT STSYQTA ST AGQ--AAMAAGLF : 213
TrhB F : GGDNAALAEQVKALGAN-----VTATGE V-----QMPAPFGEGETQ GNTVSPFQGSVAVPPTAFY : 173

*TrhB R478 : IKEGREVIAG-DKATRT RGDGTA VDSKARHAAR---KDEM FATS IITGVIT LEA PLSKSKSE MVTM KKD : 277
TrhB R27 : IREGREVIVAGSEKA TRT RGDGTA VDSKARHAAR---KDEM FATS IITGVIT LEA PLSKSKSE MVTM KKD : 278
210 Rts1 : SKSKNEA---VNIVT QODIKERHSDSDTG-----ET FATS IISGVIT SMFPAQNAED YVALH KKD : 270
TrhB pCAR1 : SSEKTEA K-----IRV KAK-EDGKSDKEEL-----SVT FATS LLSSVIT SMFPAQNAED YVALH KNE : 252
TrhB SXT : GTRARDV L---ITIRM E EVVAE EVVQDA-----PL FATS IISGTIT LLE PTHESARRE FALAN QKE : 250
TrhB R391 : GTRARDV L---ITIRM E EVVAE EVVQDA-----PL FATS IISGTIT LLE PTHESARRE FALAN QKE : 250
4262 pRS241d : QRFAGASVD---FTLQKPEK TTEESSRNRV---DLHS FATS IISGTIT LLE PTHESARRE FALAN QKE : 249
TrhB pSLT : GNGVTT Q---VITYQS V VNIQRKTFSYDAAKKGSLE FATS IISGTIT LLE PTHESARRE FALAN QKE : 248
TrhB R100 : GNGVTT Q---VITYQS V VNIQRKVFTRNEG-KQG SLT FATS IISGTIT LLE PTHESARRE FALAN QKE : 250
TrhB pNL1 : AGRISSEVSL---VGFNEGASGTGS VV KGNVTFT---DSAN FATS IISGTIT LLE PTHESARRE FALAN QKE : 286
TrhB F : GNGVTT Q---VITYQS V VNIQRKVFTRNEG-KQG SLT FATS IISGTIT LLE PTHESARRE FALAN QKE : 250

TrhB R478 : VILN-----FTMLRD N LGSAVI AAFUYI FATS IISGTIT LLE PTHESARRE FALAN QKE : 353
TrhB R27 : IILN-----FTMLRD N LGSAVI AAFUYI FATS IISGTIT LLE PTHESARRE FALAN QKE : 354
210 Rts1 : ALDRN-----FRVIRE G LAAGF LLSFHYF SEVIT FATS IISGTIT LLE PTHESARRE FALAN QKE : 345
TrhB pCAR1 : ALDRN-----FRVIRE G LAAGF LLSFHYF SEVIT FATS IISGTIT LLE PTHESARRE FALAN QKE : 327
TrhB SXT : ALDRN-----FRVIRE G LAAGF LLSFHYF SEVIT FATS IISGTIT LLE PTHESARRE FALAN QKE : 325
TrhB R391 : ALDRN-----FRVIRE G LAAGF LLSFHYF SEVIT FATS IISGTIT LLE PTHESARRE FALAN QKE : 325
4262 pRS241d : A TAAAGAGAGRRILTGIT LGSAM LLS VV LTTA GGRNKVIETCAGLVAGS---GAGT FATS IISGTIT LLE PTHESARRE FALAN QKE : 328
TrhB pSLT : VEHS-----KTYLITG F GLEARN VSSN IITRN FATS IISGTIT LLE PTHESARRE FALAN QKE : 321
TrhB R100 : VEHS-----KTYLITG F GLEARN VSSN IITRN FATS IISGTIT LLE PTHESARRE FALAN QKE : 323
TrhB pNL1 : ARSVTD-NGRLLTTHIAG LUNGAARALGGMVY LQRM LQNNRYAVSD KGFIAFG---GTEV FATS IISGTIT LLE PTHESARRE FALAN QKE : 364
TrhB F : VEHS-----KTYLITG F GLEARN VSSN IITRN FATS IISGTIT LLE PTHESARRE FALAN QKE : 323

TrhB R478 : AGAAGAGG SSAGSLSKVSNIDNSQAQYQS-----NFGALGA AGAGAAQGG--LNR VITAI QQW FATS IISGTIT LLE PTHESARRE FALAN QKE : 428
TrhB R27 : AGSAGAGG SAAGSLSKVSNIDNSTAQYQS-----NFGALGA AGAGAAQGG--LNR VITAI QQW FATS IISGTIT LLE PTHESARRE FALAN QKE : 429
210 Rts1 : AKALTAGG QAASQLFVSQSI TENVNRDGNRSGGS---YEQVMSQA QGAAS VGGALNKAIT KMTDLFEV FATS IISGTIT LLE PTHESARRE FALAN QKE : 424
TrhB pCAR1 : ARSLGAGG QAGAFNVQVY TENVTRASDDKTVAVYEQAFDSNA QSAGES AGSALEHKAIT KMTDLFEV FATS IISGTIT LLE PTHESARRE FALAN QKE : 408
TrhB SXT : AKSMAGG QAGAFDVNVVETQT---GNAGDTQL---YQVMSQA QGAAIK TGKALDRAKTDMA NMFP FATS IISGTIT LLE PTHESARRE FALAN QKE : 401
TrhB R391 : AKSMAGG QAGAFDVNVVETQT---GNAGDTQL---YQVMSQA QGAAIK TGKALDRAKTDMA NMFP FATS IISGTIT LLE PTHESARRE FALAN QKE : 401
4262 pRS241d : TNAAINAGG FAK--ALTSAAASASSNDGAETVGSV---LGG--AGAGVVGGAASAAKRAE KRA QYQV FATS IISGTIT LLE PTHESARRE FALAN QKE : 400
TrhB pSLT : GWANGAGG DGGQMERASQQAAG-----LGATAA-----YAGDVFMGIGG ASKAAQTSIA KRA QYQV FATS IISGTIT LLE PTHESARRE FALAN QKE : 391
TrhB R100 : GWANGAGG DGGQMERASQQAAG-----LGATAA-----YAGDVFMGIGG ASKAAQTSIA KRA QYQV FATS IISGTIT LLE PTHESARRE FALAN QKE : 393
TrhB pNL1 : GQATPLAGG FGRGAANTNSITGTNVVNRQKQ-----LGTGDI EAGLGE IATSGIM SKL ERF QYQV FATS IISGTIT LLE PTHESARRE FALAN QKE : 440
TrhB F : GWANGAGG DGGQMERASQQAAG-----LGATAA-----YAGDVFMGIGG ASKAAQTSIA KRA QYQV FATS IISGTIT LLE PTHESARRE FALAN QKE : 393

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TrhB R478 : SGRATTFVQTTTNTLTSR----- : 451
 TrhB R27 : SGRATTFVQKATNTLTSR----- : 452
 210 Rts1 : DPARSDFVQKVSFKFNKVTSGGNK----- : 452
 TrhB pCAR1 : DAMREDFVNRKMTKFNATTLKVNDVNQ----- : 438
 TraB SXT : DAARKDEVVTRASLATSQGGGARR----- : 429
 TraB R391 : DAARKDEVVTRASLATSQGGGARR----- : 429
 4262 pRS241d : YAGTNDEVVMEVVRK----- : 417
 TraB pSLT : GAGNEITVFQDFQKTVEEMAQEQARHRKEDENAESVFIQSVDSHMGFNTDQMLKQLGDLNQQFMSGSGQ--GHV : 470
 TraB R100 : GAGNEITVFQDFQKTVEEEMALEQSQSTAEGNPESEVNFPSAESHNGFNTDQMLKQLGNLNQQFMSGDKNPLGGD : 474
 TraB pNL1 : PTGIDIEIIFLEVEVNG----- : 458
 TraB F : GAGNEITVFQDFQKTVEEEMALERTQSRAEEDNPESVFIQSVDSHNGFNTDQMLKQLGNLNQQFMSGSGQ--GGN : 472

TrhB R478 : ----- : -
 TrhB R27 : ----- : -
 210 Rts1 : ----- : -
 TraB pCAR1 : ----- : -
 TraB SXT : ----- : -
 TraB R391 : ----- : -
 4262 pRS241d : ----- : -
 TraB pSLT : NGK----- : 473
 TraB R100 : AGKEGGNGG : 483
 TraB pNL1 : ----- : -
 TraB F : DGK----- : 475

4.4 Discussion

The movement of DNA between bacterial cells necessitates the association of cytoplasmic and membrane-associated proteins. The transmembrane Mpf complex (including 16 proteins in the R27 system) is responsible for production of the conjugative pilus, a structure proposed to contact recipient cells, and formation of the conjugative junction, through which DNA passes. The coupling protein is responsible for the membrane targeting of the relaxosome nucleo-protein complex. This study demonstrates that the coupling protein TraG interacts with the Mpf protein TrhB. The formation of TrhB homomultimers was also observed, although the exact oligomerization state was undefined. The TraG-TrhB protein interaction suggests a mechanism by which the cytosolic DNA molecule is targeted to the transmembrane apparatus for export to recipient bacteria.

TrhB and TraG homologues are found in all known type IV secretion systems, including the conjugative systems (Christie, 2001; Lawley *et al.*, 2003a). It is not surprising that conserved elements are needed to mediate the Mpf complex/coupling protein interaction. TrhB has previously been identified as a mating pair formation (Mpf) protein, since transposon mutagenesis of *trhB* renders R27 transfer deficient and resistant to Hgal, an H-pili specific bacteriophage (Lawley *et al.*, 2003a). This mutant has been complemented for both transfer and pilus production by producing TrhB_{His6} *in trans* (Lawley *et al.*, 2003a). The observed TrhB homotypic interaction could be mediated through the predicted coiled-coil region spanning residues 77-124 (Figure 4-2), as a construct representing the first 133 amino acids of TrhB interacted with full-length TrhB. Coiled-coils are assemblies of α -helices, and this structure has been found extensively in secreted components of T3S systems (Delahay and Frankel, 2002). A coiled-coil structure (Figure 4-6) is also predicted to occur in numerous TrhB homologues, including TraB (F, R391, pSLT, R100, SXT, pNL1) and TrhB (R478 and

pCAR1). Although computational predictions for coiled-coil domains are not always accurate, the presence of this feature in a large number of proteins from a single family is indicative of a correct prediction (Lupas, 1996). Furthermore, this region of TrhB is completely free of proline residues, which prohibit the formation of helical structures (Muller *et al.*, 2000).

The membrane topology of TraG and TrhB are unknown. However, it is predicted that TraG is polytopic, with two closely spaced transmembrane (TM) domains at the N-terminus and a short periplasmic loop. This is based upon sequence analysis of TraG and the known membrane topology of other coupling proteins. TMHMM predicted two TM domains at residues 19-41 and 45-64 of TraG (Figures 2-6 and 4-2), likely resulting in cytoplasmic localization for both the short N-terminal segment and the large C-terminal domain containing nucleotide triphosphate-binding motifs. TraG (RP4), TraD (F), and VirD4 (Ti) all have two TM domains at the N terminus separated by a periplasmic domain (Das and Xie, 1998; Lee *et al.*, 1999; Schroder *et al.*, 2002). TrhB is a member of the F-like TraB family of proteins, which are each predicted to contain an N-terminal transmembrane domain and high concentration of proline residues between a coiled-coil domain and the highly conserved C-terminal domain (Figure 4-6). TrhB is predicted to be anchored in the inner membrane by a singular N-terminal TM domain (residues 12-34), and is likely bitopic, with a large C-terminal portion extending into the periplasm (Figure 4-6). The TrhB homologue TraB from the F plasmid (27.2% identity over alignment length = 482, see section 4.2.7) has been predicted to have a large periplasmic domain following the TM domain at residues 13-33 (Frost *et al.*, 1994). TraB was found to be an integral membrane protein, associating with the outer membrane in *tra*⁺ cells and to the inner membrane in *traV* and *traK* mutant cells (Harris *et al.*, 2001). The VirB10 protein required for T-DNA transfer from *A. tumefaciens* is also similar to TrhB (20.4% identity over alignment length = 460) and was found to be an integral inner

membrane protein with the bulk of the protein existing in the periplasm, as revealed by *phoA* fusions (Ward *et al.*, 1990). The co-localization of TraG and TrhB in the inner membrane would in part facilitate an interaction between these two transfer proteins. Neither TraG nor TrhB are predicted to have a cleavable N-terminal signal sequence using the parameters outline by von Heijne (von Heijne, 1986).

Both TrhB and TraB (F) have proline-rich regions (Figure 4-6), indicative of extended structures and protein-protein interaction domains. There is only one proline residue present in the first 134 amino acids (0.7%) of TrhB, whereas the remaining 318 residues include 22 prolines (7%). TraB (F) has a 7% total composition of proline and is comprised of 35% proline from amino acids 135-183 (Anthony *et al.*, 1996; Harris *et al.*, 2001). Similarly, TrhB has a 39 residue segment from 135-173 that is 17.9% proline. Considering that TrhB₁₋₂₂₀, and not TrhB₁₋₁₃₃, interacted with TraG, this region may be involved in TraG interaction. Related proteins in the IncP (TrbI, 20.3% identity over alignment length = 518), Ti (VirB10) and IncW (TrwE, 19.2% identity over alignment length = 536) systems all lack predicted coiled-coil domains, yet are also enriched for proline. TrbI is 6.7% proline with a domain from amino acids 88-129 that is 26% proline, VirB10 from pTiC58 is 7.8% proline and 28% proline from 61-114 and TrwE is also 7.8% proline with a domain from 80-137 comprised of 31% proline. Das and Xie found that a LexA fusion of a peptide representing amino acids 47-277 of VirB10 pTiC58 was positive in a yeast two-hybrid analysis with full-length VirB10, whereas a LexA fusion of amino acids 167-377 was negative (Das and Xie, 2000). The inferred homotypic interaction domain of VirB10 also coincides with the proline-rich segment of this protein. TrhB homotypic interactions may solely be due to the coiled-coil segment, however, contributions from the proceeding proline-rich domain may also facilitate this interaction, as indicated by the TrhB homologues lacking the coiled-coil structure. Interaction between TraG and TrhB may also be determined through the TrhB proline-rich domain,

since the N-terminal half of TrhB was capable of supporting this interaction.

Furthermore, the conserved proline-rich domain present in TrhB-related transfer proteins (TrbI, TrwE and VirB10) may enable binding to the respective coupling protein.

Yeast two-hybrid and fractionation studies of the F plasmid Mpf proteins TraB, TraK and TraV revealed that these proteins form a transmembrane complex extending from the inner membrane to outer membrane (Harris *et al.*, 2001). The R27 Mpf protein TrhK has sequence similarity to TraK, and likewise, R27-encoded TrhV is similar to TraV, the TraK-associated lipoprotein (Harris and Silverman, 2002; Lawley *et al.*, 2003a).

Considering the sequence conservation and functional similarities of TrhB, TrhK and TrhV to the F plasmid counterparts, and the similar domain structure of TrhB and TraB, it is plausible that TrhB, TrhK and TrhV enter into a similar transmembrane complex. In this complex TraB may span the periplasm to associate with TraK in a manner similar to the energy-transducing TonB, which has a proline-rich domain that allows extension across the Gram-negative periplasmic space for interaction with outer membrane proteins involved in active transport (Chang *et al.*, 2001; Skare *et al.*, 1993). It was previously found that both TraK and TrhK sequences are similar to members of the outer membrane secretin protein family (Lawley *et al.*, 2003a), and there are preliminary data showing that TraK forms high molecular weight, SDS-resistant complexes (unpublished data). The B/K/V Mpf complex may represent the core transenvelope complex for T4S of H and F plasmids.

Secretins are secretory proteins which form outer membrane-associated ring structures. The secretin subunit PilQ forms a dodecameric ring in the outer membrane *Neisseria meningitidis*, and is required for the secretion of extracellular type IV pilus structures in *N. meningitidis*, *N. gonorrhoeae*, and *Pseudomonas aeruginosa* (Collins *et al.*, 2001; Drake and Koomey, 1995; Martin *et al.*, 1993). Additional examples of secretins are present in type II (*Klebsiella* PulD) and T3S systems (*Shigella* MxiD,

Salmonella InvG, *Yersinia* YscC and *Erwinia* HrcC) (Guilvout *et al.*, 1999; Kaniga *et al.*, 1994; Kim *et al.*, 1997; Koster *et al.*, 1997; Schuch and Maurelli, 2001). Notably, a tripartite transmembrane interaction between the inner membrane MxiJ, outer membrane secretin MxiD and MxiM lipoprotein was also observed in the *Shigella* T3S apparatus (Schuch and Maurelli, 2001). This may represent an analogous structure to a transenvelope B/K/V Mpf complex.

Although TraG interacts with TrhB, the coupling protein is not a required component of the transmembrane protein complex for pilus assembly since a R27 *traG* mutant-containing cell produced H-pili (Lawley *et al.*, 2002a). Alternatively, TrhB, TrhK and TrhV were each essential for pilus biogenesis (Lawley *et al.*, 2003a). The data presented in this chapter indicate that in the absence of TraG, TrhB is stably maintained, and thus likely still present in a B/K/V Mpf complex. However, when TraG is present, the B/K/V Mpf complex may be adapted for export of DNA; hence there may be two populations of transport apparatus, those with or without the coupling protein, and each capable of a different function. This may also be true in the T-DNA transfer system, as *virD4* mutants of pTiC58 still produce T-pili (Lai *et al.*, 2000).

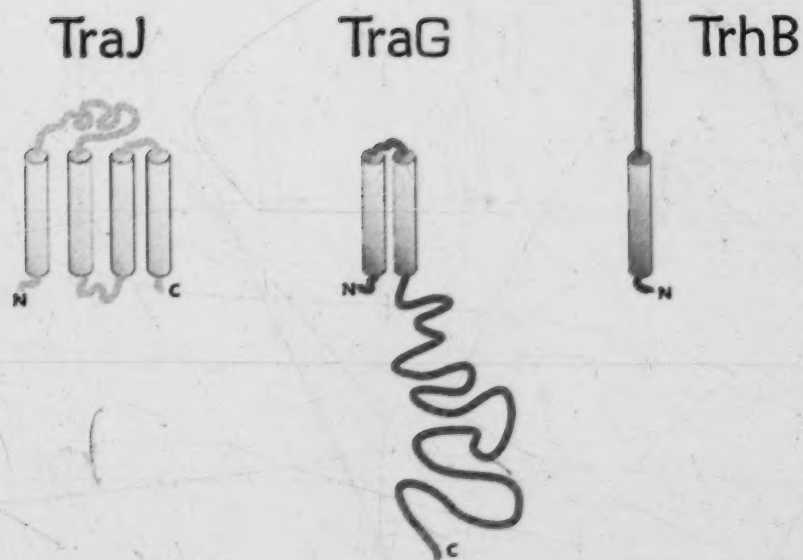
These observations allow a model for the macromolecular assembly of transfer proteins into a transmembrane pore for DNA translocation to be proposed (Figure 4-7). Besides interacting with the TraG coupling protein, TrhB may interact with an outer membrane-associated secretin, predicted to be TrhK. Considering the structural data for the IncW coupling protein TrwB (Gomis-Ruth *et al.*, 2001) and the known arrangement of secretins, it is possible that TraG and TrhK each form ring structures in the inner and outer membranes, respectively. If so, TrhB molecules may form a central ring structure spanning the periplasmic space, as TrhB was shown here to minimally form a homodimer and contains coiled-coil and proline-rich features which likely result in an extended conformation. This predicted superstructure would correlate to the stacked

ring superstructures having 'cylindrical symmetry' observed for both type III secretion and flagellar motor transmembrane structures (Blocker *et al.*, 2001; Kubori *et al.*, 1998; Thomas *et al.*, 2001).

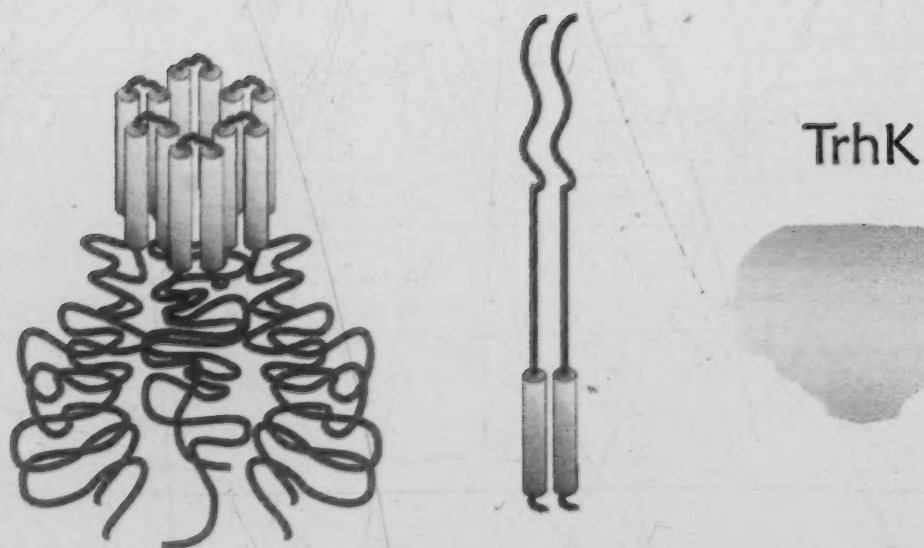
The stacked ring model for the conjugative apparatus necessitates that TrhB is able to span both the inner membrane and periplasmic space (through transmembrane and rigid proline-rich domains, respectively) to interact with the outer membrane secretin TrhK. Deletion constructs of TrhB used in conjugation with TraG in the BTH assay, however, indicated that the proline-rich domain is required for TrhB-TraG interactions (Figure 4-1). The predicted topology for TraG indicates that only a loop of four amino acid residues is present in the periplasmic space, providing a small substrate for interaction with TrhB proline-rich domain. It is more plausible that that TrhB and TraG interact through their inner membrane-spanning domains (as illustrated in Figure 4-7), because these are the only significant segments between these proteins which co-localize to the same subcellular compartment.

Figure 4-7. Model for the assembly of a pore-like transfer apparatus that traverses the cell envelope. The predicted transmembrane domains of TraJ, TraG, and TrhB monomers are represented as cylinders, with N- and C-termini specified (A). The rigid conformation illustrated for TrhB is predicted from the high proline content in the primary sequence. The predicted multimeric state of TraG and the minimal multimeric state of TrhB are shown (B). TrhK, predicted to be the H-type secretin molecule (Lawley *et al.*, 2003a) is represented as multimeric ring structure, modeled after the three dimensional structure of *Neisseria meningitidis* dodecameric secretin PilQ (Collins *et al.*, 2003). A possible assembly of these proteins into a cell envelope-spanning superstructure is presented (C). Specific interactions are proposed or known to occur between the relaxase-TraG, TraG-TrhB, TrhB-TrhK (see text). The role of TraJ is unknown, but it is a conserved member of the H-type transfer apparatus.

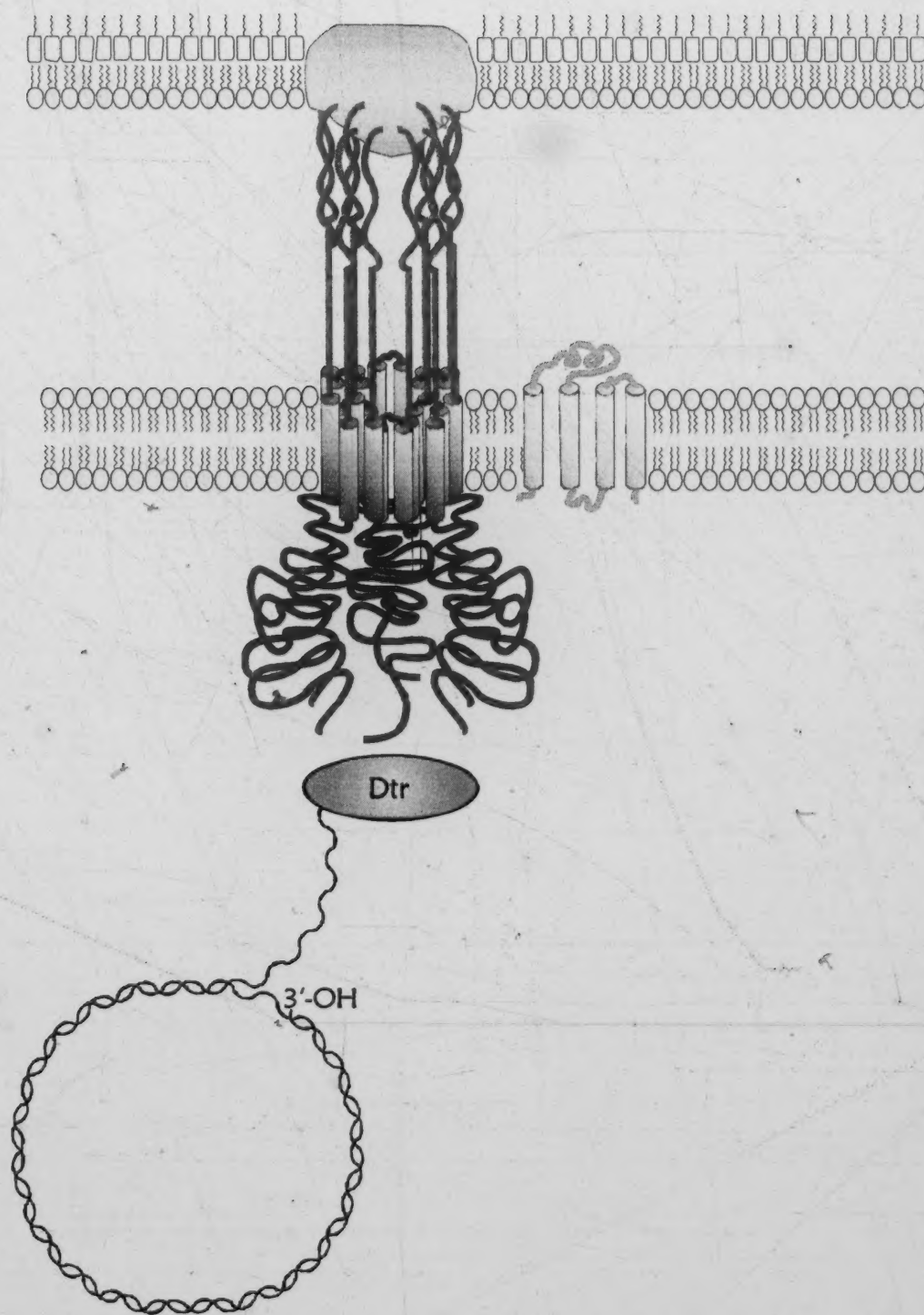
A.



B.



C.



Chapter 5

General Discussion

5. General Discussion

5.1 Genome evolution

Horizontal gene transfer has been suggested to "uproot" the tree of life because of the trans-domain transfer of genetic determinants, a process which may also challenge aspects of the Darwinian theory of evolution (Doolittle, 2000). Rather than being three independent branches, the three domains of life are now realized to resemble an interconnected web because of transfer events between different genomes, demonstrating that genetic inheritance is not confined to species-specific lineages. As genomes evolve through the mechanistic process of horizontal gene transfer, dramatic phenotypic changes can occur after a single transfer event, however the principles of 'survival of the fittest' still apply. Not all transfer events will be beneficial to the recipient, and selection of transferred genes no doubt still occurs. Genes which are unlikely to be transferred and successfully maintained include the core set of genes required for basic cellular processes (RNA, tRNA, chromosome replication, transcription, and translation proteins), as recipients would already encode such features, and the acquired determinants might be unable to interact with the native complement of core factors (Jain *et al.*, 1999; Lawrence and Hendrickson, 2003). In contrast, those determinants offering novel phenotypic traits (that are able to function alongside resident features) might be selected for horizontal transfer (Lawrence and Roth, 1996). This process is exemplified by the transfer of resistance determinants.

Conjugative resistance plasmids, in addition to being a vector for horizontal gene transfer, also represent distinct genetic units which are subject to processes of acquisition, mutation, and deletion. Through the comparative sequence analysis of IncHI plasmids R27, pHCM1, and R478, a conserved set of determinants for plasmid replication, partitioning, and transfer were revealed. These features are likely conserved

because each represent core determinants necessary for vertical and horizontal transmission. In contrast, coding sequences which result in resistance phenotypes are not strictly required for the maintenance of plasmids in the bacterial gene pool. The resistance genes/operons are therefore classified as accessory determinants, and represent exchangeable modules which enhance the phenotypic advantages imparted to the host bacteria.

The conjugative transfer determinants of the IncHI plasmids (or an ancestral sequence) were acquired from different sources. A sequence comparison with numerous other genetic elements demonstrated that the Mpf genes are of a different lineage than the Dtr/coupling determinants. Despite this bifurcated history, an interaction between these different classes of proteins is necessary for the assembly and function of conjugative apparatus. This was revealed by a biochemical characterization of protein-protein interactions and a fluorescent visualization of the conjugative protein apparatus. The ancestral sequences of the H plasmid transfer system were possibly independent functional modules that separately supported pilus production (Mpf) and DNA metabolism (Dtr), but which have converged and co-evolved to establish a singular functional module for conjugative transfer. The adaptation of two ancestrally distinct modules is not only evident in the genetic sequences and organization of H plasmids, but also at the protein interface between these two modules.

5.2 The multifunctional Mpf proteins

Classification of conjugative transfer proteins as Mpf determinants is partly based upon the inability of mutants to support both plasmid transfer and pilus production. Conversely, those mutations which affect only transfer but not assembly of pili are classified as Dtr or coupling determinants. The logic behind this classification scheme is

that the Mpf proteins can assemble and function in the absence of the Dtr/coupling determinants. This has been demonstrated through not only the detection of pili on Dtr/coupling mutants (Lawley *et al.*, 2002a), but also by the continued production and stability of Mpf protein TrhB (Figure 4-5), and the visualization of TrhC-associated protein subcomplexes (Figure 3-6) in the absence of relaxase or coupling proteins. The ancestral function of the Mpf module may have been solely pilus production, but was subsequently adapted for conjugative transfer by addition of the Dtr and coupling determinants.

The phenotypic advantages of a bacterial cell producing pili using the Mpf module include the ability to enter into biofilms. Ghigo has demonstrated that cells expressing one of a number of different types of Mpf systems (including those from IncF, I, H2, X, T, N, and W groups) resulted in the formation of biofilms, a process likely initiated by the respective conjugative pili (Ghigo, 2001). This correlates with the observed temperature sensitivity of the H plasmid Mpf system, as pili are produced at lower temperatures, possibly promoting the formation of biofilms under environmental conditions. Therefore an ancestral form of the Mpf module may have existed for the export and assembly of extracellular pili, which were responsible for adhesion to environmental substrate and intimate cell-cell association resulting in biofilm formation. The ability of the ancestral Mpf module to export substrate (i.e. pilin subunits) across the cell envelope and to make contacts with neighbouring cells (via the pilus) may have then been 'exploited' by the Dtr and coupling determinants for conjugative transfer of cytoplasmic DNA molecules.

5.3 H-type Dtr/coupling determinants

The TraJ transfer determinant was previously thought to be unique to IncHI plasmids, and was classified as a Dtr component (Lawley *et al.*, 2002a). After PSI-BLAST analysis of the R478-encoded TraJ sequence, sixteen potential orthologues were identified in

both plasmid and chromosomal genomes (Table A-1). Although there is modest sequence identity between these sixteen sequences and TraJ (13-31% local identity), each are ~220 residues in length, each contain four predicted transmembrane domains, and each have conserved subdomains, including a tandem of positive residues between the third and fourth transmembrane domains (Figure 2-7). The precise cellular function of TraJ is unknown, however the discovery of related sequences in numerous other genomes suggest that TraJ is an important Dtr determinant.

The IncHI Dtr and coupling determinants Tral and TraG were not considered unique to the IncHI complex because they contain conserved motifs found in the respective IncP and IncW-encoded Dtr and coupling determinants, such as relaxase and Walker NTP-binding motifs (Lawley *et al.*, 2002a; Sherburne *et al.*, 2000). An up-to-date PSI-BLAST analysis of R478-encoded relaxase and coupling proteins identified numerous coding sequences which have sequence identity to Tral or TraG outside of these motifs (Figures 2-5 and 2-6), therefore more closely related orthologues than the P and W determinants exist. Notably, eleven of the genomes which encode IncHI Tral and TraG-related sequences, also encode a TraJ orthologue (Table 2-2). This indicates that *tral*, *traG*, and *traJ* are genetically linked in numerous genomes, and that they might have been inherited from a common ancestor. For the purpose of this discussion, these three genes will collectively be designated the H-type Dtr/coupling determinants, representing a novel phylogenetic group of transfer sequences.

The Mpf module of the IncHI plasmids is related to the F-type Mpf module, yet the H-type Dtr/coupling determinants are distinct from the F-type Dtr/coupling sequences. In particular, the Mpf genes of R478 are closely related to the sequences encoded on pRS241d, pNL1, pSLT, and *V. vulnificus* (Figure 2-4), but these F-type genomes do not include H-type *tral-G-J* sequences. These observations suggest that the IncHI plasmid

transfer system evolved from two different lineages which converged to form a novel module that is functional for plasmid transfer.

Just as the IncHI Mpf module might have had a different cellular function prior to being adapted for conjugative transfer, the function of an ancestral H-type *tral-J-G* module (in the absence of Mpf functions) may have been related to a general DNA processing reaction. The conjugative relaxases such as Tral can participate in the most basic DNA metabolic functions: that of cutting and rejoining and therefore serving as both endonuclease and ligase. These are each essential features of both DNA recombination and replication (i.e. during lagging strand synthesis) reactions, and furthermore, type I topoisomerase enzymes similarly nick one strand of a DNA duplex and serve as a ligase (Cavalier-Smith, 2002). The coupling protein TraG has also been predicted to have a role in DNA metabolism, as revealed by the structure of IncW coupling protein TrwB (Gomis-Ruth *et al.*, 2001). The homohexameric structure of TrwB resembles that of DNA ring helicases which act as pumps for single DNA strands passing through the central channel, thereby serving as a motor for DNA translocation. TraJ contains no known functional motifs, but may be required for the coordinated function of Tral and TraG.

5.4 Functional adaptation of the Mpf and Dtr determinants

The versatile role of the coupling protein is not only indicated by its predicted ability to serve as an inner membrane pore for transfer of the single-stranded transfer intermediated, but by many clear examples of chimeric transfer systems comprised of non-cognate coupling proteins (Table 5-1). Some transferable elements encode only one of the two conjugative transfer modules, and mobilizable plasmids such as RSF1010 have only the Dtr module, and lack the Mpf module and a coupling protein. To support conjugative transfer, the remaining determinants must be supplied by a non-

Table 5-1. Chimeric functional groups capable of mobilizing DNA. The plasmid source or lineage of the different classes (Mpf, coupling, Dtr/Mob) of conjugal transfer proteins are listed. For example, in this study R478 has H-type Dtr and coupling determinants, but F-type Mpf determinants. All other examples represent experimental systems where a functional apparatus has been reconstituted from different lineages of transfer determinants.

Mpf	Coupling	Dtr/Mob	Reference
F	H	H	This study
N	W	W	Llosa <i>et al.</i> , 2003
W	N	N	Llosa <i>et al.</i> , 2003
X	W	W	Llosa <i>et al.</i> , 2003
X	N	N	Llosa <i>et al.</i> , 2003
P	P	RSF1010	Lessl <i>et al.</i> , 1993
W	P	RSF1010	Cabezón <i>et al.</i> , 1994
W	P	ColE1	Cabezón <i>et al.</i> , 1994
W or F	F ^a	RSF1010	Sastre <i>et al.</i> , 1998
W or F	F	ColE1	Sastre <i>et al.</i> , 1998
Ti	Ti	RSF1010	Beijersbergen <i>et al.</i> , 1992
P	P	pBHR1	Szipirer <i>et al.</i> , 2000
P	P	pBGR1	Seubert <i>et al.</i> , 2003a
F, I, N, P, W	CloDF13	CloDF13	Cabezón <i>et al.</i> , 1997
X	CloDF13	CloDF13	Strauch <i>et al.</i> , 2003

a. An F plasmid TraD construct with a 140 residue C-terminal truncation was used

cognate system. Therefore, the RSF1010 Dtr components must interact with an unrelated coupling protein (Table 5-1). Other mobilizable plasmids such as CloDF13 encode both the Dtr and coupling determinants, but lack the Mpf module. In this instance, the CloDF13 coupling protein must interact with an unrelated Mpf module (Table 5-1). In addition, interactions between TrhB-related proteins and non-cognate coupling proteins have also been demonstrated (Table 5-1), which also confirms the results presented in Chapter 4 (Llosa *et al.*, 2003). These observations verify that interactions between transfer determinants from different lineages occur, and the coupling protein plays a central role in adaptation of the different transfer modules.

Select T4S effector translocation systems may also depend upon coupling proteins for substrate recruitment in a fashion similar to relaxase-DNA recruitment during conjugative transfer. Each of the *A. tumefaciens* Vir, *H. pylori* Cag, and *L. pneumophila* Dot/Icm systems require a coupling protein, whereas the T4S systems encoded by *Bordetella*, *Brucella*, and *Bartonella* spp. apparently function without a coupling protein (Cascales and Christie, 2003). It is possible that in these systems in which the coupling protein is not required for substrate translocation that the nature of the substrate does not require a coupling protein to interact with the transenvelope type IV secretion apparatus.

5.5 A transenvelope structure for macromolecular transport

The term 'conjugative apparatus' might be hyperbole considering that a transenvelope structure has never been visualized through traditional microscopy. Notably, the IncP relaxosome has been visualized under *in vivo* conditions (Pansegrau *et al.*, 1990) and a membrane fraction (of intermediate density between the inner and outer membrane fractions) containing the IncP Mpf proteins was observed (Grahm *et al.*, 2000). This latter observation supports the hypothesis that conjugal transfer proteins

indeed form a transenvelope structure, and this thesis has provided additional cytological evidence (Chapter 3). The visualization of fluorescent TrhC-GFP foci in live bacterial cells represents an indirect visualization for a macromolecular assembly of transfer proteins. These studies indicated that a large network of proteins is responsible for foci formation, and that ATPase function of TrhC is dispensable for complex assembly.

The Mpf, coupling, and Dtr modules are individually capable of distinct functions, such as DNA processing and pilus production; however, the cellular function of the conjugative apparatus is dependent upon the interaction and coordinated operation of these modules. Using the TrhC-GFP fusion as a label for the conjugative apparatus, mutations in Mpf, Dtr and coupling determinants each affected the assembly of this machine. TrhC is classified as an Mpf protein, yet non-Mpf proteins such as TraJ were critical for assembly of the TrhC-GFP foci. In addition, the interaction between TraG and the Mpf protein TrhB may account for the observed influence of TraG on TrhC-associated complex assembly, of which TrhB is a member. These observations highlight the relationship of the different structures that compose the transfer apparatus: distinct subassemblies and complexes may be observed, but coordination of all the elements as a singular unit may ultimately be required for function.

5.6 Future Directions

The three-dimensional structure of individual conjugative transfer proteins has been obtained (Gomis-Ruth *et al.*, 2001; Guasch *et al.*, 2003), and has in each instance provided a wealth of insight into the mechanism of DNA processing and transport. The data presented in this thesis indicates that the different transfer protein modules are dependent upon each other for assembly and function of the conjugative apparatus. It might be possible to purify and obtain structural information for heteromeric protein

complexes, and ultimately identify the architecture of the conjugative apparatus. Of particular interest would be the precise association of the coupling proteins and TrhB homologues at the inner membrane, and if the TrhB homologues extend through the periplasm to interact with an outer membrane-associated secretin-type transfer protein.

A detailed characterization of the conjugative apparatus might also aid in determination of the true substrate of conjugative transfer and the key proteins which make up the transenvelope pore. It is plausible that one or both of the transfer DNA strand or covalently attached relaxase are transferred to recipient cells. The majority of T4S systems seem geared for the export of protein substrates, including the *A. tumefaciens* system which transfers the VirD2 relaxase in association with the T-DNA. Furthermore, the precise roles of the numerous ATPase transfer proteins encoded by each conjugative system during substrate transport are still unknown. The ATP-binding motifs of TrhC were not required for assembly of TrhC-GFP-associated complexes, however, function during pilus assembly and/or DNA translocation remain possibilities.

An interesting paradox concerning the evolution of conjugative plasmids is that while being a vehicle for horizontal transfer of genetic information, they are also subject to acquisition of horizontally-transferred coding sequences. This is indicated by the large number of genes and operons encoded on the sequenced IncHI plasmids which were attributed to different origins. In particular, the IncHI transfer determinants have been acquired from at least two lineages. The ancestral history of the prototypic plasmid systems might be further revealed using the growing amount of sequence data, for both whole genomes and individual plasmids. The observation of chromosomal sequences which are related to plasmid-transfer determinants encoding DNA-processing and membrane-targeting features is intriguing and might suggest that a widespread and uncharacterized mechanism for DNA transport is encoded in multiple microbial genera. In addition, an increasing number of T4S effector translocation systems will undoubtedly

be uncovered in the genome data from both pathogenic and non-pathogenic bacteria.

The relationship between these transport systems and true conjugative systems may also be discovered, suggesting a mechanism for the evolution of these analogous protein machines.

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Appendix

Table A-1. Predicted coding sequences of plasmid R478. Each amino acid sequence was compared against the non-redundant protein database at the National Center for Biotechnology Information (NCBI) using BLAST or PSI-BLAST and similar sequences from R27, pHCM1, and any other genetic element are reported.

Gene/Protein	Co-ordinates ^a	Amino acids	pHCM1 / R27 Homologue	% Identity/Range (E value) ^b	Other Homologues	% Identity/Range (E value) ^b	Accession number
SMR0001 RepHIA	1-876	291	HCM1.64 (RepA2) R0036 (RepHIA) HCM1.54 (RepA) R0050 (RepHIB)	86%/289 (e-134) 86%/289 (e-134) 36%/288 (e-41) 36%/288 (e-41)	replication protein A (Versinia pMT1) RepA (pHCM2) RepA (Vir plasmid, S. choleraesuis) RepA (pSLT) RepFIB (F plasmid) RepA (pO157) RepA (P1 phage) RepA (Ris1) RepA2 (pS268a, Serratia sp.)	42%/286 (2e-61) 42%/286 (3e-61) 42%/280 (e-60) 42%/280 (2e-60) 43%/273 (9e-60) 44%/267 (3e-59) 44%/269 (2e-59) 41%/278 (4e-52) 41%/115 (4e-19)	AAC13203 NP_589603 NP_073241 NP_490513 AA71886 NP_052830 BVBPRA NP_639962 AAL84748
SMR0004 TmA	2025-2378	117	HCM1.67 (TmA) R0034 (TmA)	65.8%/117 (3.9e-28) 65.8%/117 (3.9e-28)	TraA (Vibrio cholera, SXT) TraA (R391) TmA (pCAR1, Pseudo. resinovorans) 20631 (Vibrio vulnificus)	18%/86 (0.71) 18%/86 23%/78 18.5%/146	AAL58683 AAM08062 NP_758680 NP_762584
SMR0005 TmL	2396-2746	116	HCM1.69 (TmL) R0033 (TmL)	71.6%/116 (4.9e-36) 71.6%/116 (4.9e-36)	RspH4259 TmL (pCAR1) 20632 (Vibrio vulnificus)	16.8%/137 17.2%/122 16.6%/145	ZP_00008310 NP_758669 NP_762585
SMR0006 TmE	2758-3546	262	HCM1.70 (TmE) R0032 (TmE)	74.3%/261 (2.6e-76) 74.3%/261 (2.6e-76)	TmE (pCAR1) TraE (SXT) TraE (R391) ORF208 (Ris1) TraE (pED208, S. typhimurium) TraE (R100) TraE (pSLT) RspH4260 20633 (Vibrio vulnificus)	26%/170 (2e-10) 26%/167 (1e-14) 25%/115 (e-5) 25%/117 (9e-5) 26%/173 (3e-4) 25%/147 (0.018) 22%/171 17.9%/182 18.2%/292	NP_758670 AAL59679 AAM08060 NP_640168 AAM99705 NP_052949 NP_490565 ZP_00008311 NP_762586
SMR0007 TmK	3546-4817	423	HCM1.71 R0031 (TmK)	79.7%/423 (1.6e-100) 79.7%/423 (1.6e-100)	ORF209 (Ris1) TraK (R391) TmK (pCAR1) s048 (SXT) RscC (P. fluorescens) HrcC (P. syringae) RspH4261 20634 (Vibrio vulnificus)	24%/376 (8e-11) 26%/253 (1e-06) 25%/258 (6e-8) 26%/253 (8e-7) 25%/124 (0.013) 32%/68 (0.15) 25.7%/113 19.5%/159	NP_640169 AAM08021 NP_758671 AAL59718 AAK81929 AAL34756 ZP_00008312 NP_762587
SMR0008 HidO	4819-5280	153	HCM1.72 R0030	45.1%/144 (4.6e-18) 45.1%/144 (4.6e-18)			
SMR0009 TmB	5270-6625	451	HCM1.73 (TmB) R0029 (TmB)	86.5%/452 (1.5e-128) 86.5%/452 (1.5e-128)	ORF210 (Ris1) TmB (pCAR1) TraB (SXT) TraB (R391) RspH4262 (Rhodobacter sphaeroides) TraB (pSLT) TraB (R100) TraB (pED208)	29%/462 (4e-42) 27%/464 (2e-36) 39%/1210 (3e-36) 39%/210 (3e-36) 31%/228 (2e-18) 31%/207 (6e-18) 30%/207 (5e-17) 24%/453 (6e-14)	NP_640170 NP_758672 AAL59682 AAM08009 ZP_00008313 NP_490567 NP_052951 AAM90707

SMR0010	6633-7115	160	HCM1.74 R0028	53.3%/152 (1.2e-25) 53.3%/152 (1.2e-25)	Trab (pNL1) 20635 (<i>Vibrio vulnificus</i>)	25%/427 (4e-11) 23.2%/478	NP_049165 NP_762588
SMR0011	7129-7986	285	HCM1.75 R0027	77%/285 (e-125) 82%/250 (e-121)	DsbC (R391) disulphide bond isomerase (SXT) ORF212 (Rts1) DsbC (pCAR1)	26%/195 (9e-12) 26%/195 (9e-12) 22%/240 (0.014) 23%/197	AAM08034 AAL59715 NP_640172 NP_758681
SMR0012	7996-8946	316	HCM1.76 (TrhV) R0025 (TrhV)	80%/316 (2.3e-88) 80%/316 (2.3e-88)	Trav (SXT) HtdD (R391) ORF211 (Rts1)	54%/48 (0.019) and 23%/214 (1.6) 54%/48 (0.020) and 32%/67 (0.074) 36%/66 (0.52) and 36%/74 (0.98)	AAL59672 AAM08037 NP_640171
SMR0013	8955-11636	893	HCM1.77 (TrhC) R0024 (TrhC)	83.9%/893 (0) 83.9%/893 (0)	TrhV (pCAR1) RspH4263 20636 (<i>Vibrio vulnificus</i>)	32%/61 (0.078) 16.1%/273 20.9%/148	NP_758673 ZP_00008314 NP_762589
SMR0014	15475-16728	417	HCM1.86 R0020 (ParA)	89.4%/416 (1.6e-143)	TrhC (pCAR1) TrhC (SXT) TrhC (R391) ORF213 (Rts1) RspH4264 TrhC (pSLT) TrhC (F) TrhC (R100) TrhC (pNL1) TrhC (R388) 20637 (<i>Vibrio vulnificus</i>)	28%/840 (6e-85) 27%/868 (7e-90) 27%/868 (7e-90) 26%/870 (3e-75) 24%/575 (9e-40) 22%/901 (5e-29) 22.9%/900 (2.7e-9) 22%/902 (5e-28) 22%/532 (8e-25) 21%/178 (7e-6) 18.6%/451 24.1%/382	NP_758682 AAL59681 AAM08001 NP_640173 ZP_00008315 AAL23492 AAB61935 NP_052960 NP_049162 CAC78982 NP_762590 NP_762591
SMR0015	16725-17729	334	HCM1.87 R0019 (ParB)	72.1%/330 (1.8 e-88) 72.1%/330 (1.8 e-88)	VCA1115 (<i>Vibrio cholera</i>) ParA (pMT-1, Y. pestis) ParA (phage P7) ParA (phage P1) ParA (pSLT)	27.6%/395 (5.6e-23) 28%/234 (2e-10) 28%/232 (2e-10) 24%/264 (2e-10) 28%/222 (6e-10)	AAF97006 AAC82736 S06099 BVECPA NP_490542
SMR0016	c17791-18180	129	HCM1.88c R0018 (TrhZ)	80.5%/128 (2.3e-38) 80.5%/128 (2.3e-38)	ParB (P1) ParB (<i>Shigella flexneri</i> pCP301) ParB (pSLT) ParB (Rts1)	28%/303 (6e-21) 28%/296 (2e-20) 28%/297 (2e-19) 27%/289 (3e-18)	AAA99231 AAL72312 AAL23465 BAB93856
SMR0017	c18177-18698	173	HCM1.89C	57.7%/168 (2.9e-37) 58.3%/168 (1.8e-37)	ParB (<i>S. choleraesuis</i> vir plasmid) ParB (<i>Yersinia pestis</i> pMT1) ParB (P7) SopB (pO157 and F)	27%/262 (1e-15) 26%/295 (8e-15) 27%/291 (3e-16) 24%/161 (6e-5)	BAB20544 CAB55249 S06100 NP_052641
SMR0018	c18691-19527	278	HCM1.90c R0016	45.1%/237 (3.7e-35) 45.1%/237 (3.7e-35)			

SMR0019 TrhO	c19524-20471	315	HCM1.91c R0015 (TrhO)	71.7%/314 (1.2e-95) 71.7%/314 (1.2e-95)	Ser/Thr phosphatase (PphB, pSLT) ORF75 (pCAR1) * and numerous phosphatases (1G5B)	25%/120 (0.72) 34%/67 (0.019)	AAI21787 NP_758617
SMR0020 ParM	20835-21872	345	HCM1.92 R0014 (ParM)	80.2%/344 (7.7e-101) 80.2%/344 (7.7e-101)	Z2979 (E. coli O157:H7) Sida (pB171, EPEC) Sida (Shigella flexneri pCP301) ParM (R1) ParA (pCTXM3, Citrobacter freundii)	36%/317 (2e-45) 37%/324 (4e-44) 35%/330 (4e-40) 31%/316 (2e-35) 31%/327 (e-28)	AAG56911 BAAB4903 AAL72301 PDB id: 1MWK NP_774986
SMR0021 ParR	21884-22522	212	HCM1.93 R0013 (ParR)	41.4%/222 (1.2e-19) 41.4%/222 (1.2e-19)			
SMR0022 HidA	22925-23377	150	HCM1.94 (HidA) R0012 (HidA)	84.6%/149 (2e-51) 84.6%/149 (2e-51)			
SMR0023 HidF	23367-23792	141	HCM1.95 (HidF) R0011 (HidF)	50.7%/142 (2e-24) 50.7%/142 (2e-24)			
SMR0024 HidK	23801-24355	184	HCM1.96 (HidK) R0010 (HidK)	51.3%/187 (2.6e-34) 51.3%/187 (2.6e-34)	ORF131 (pCAR1)	37%/45 (9.1) (90-134 vs. 1276-1321)	NP_758674
SMR0025	24475-28779	1434	HCM1.97 R0009	83%/1428 (0) 83%/1428 (0)	ORF136 (pCAR1) ORF131 (pCAR1)	27%/421 (e-29) (983-1405 vs. 48-457) 21%/402 (3e-19) (511-910 vs. 1585-1961)	NP_758679 NP_758674
SMR0026 ThpP	29057-29569	170	HCM1.98 (ThpP) R0008 ThpP	77.6%/179 (2.8e-52) 77.6%/179 (2.8e-52)	ORF215 signal peptidase (Rts1) TrpF (SXT) TrnF (R391) PA1303 (Pseudomonas aeruginosa) TrnF (pCAR1) Signal peptidase I (B. cereus)	33%/129 (5e-9) 24%/160 (4e-7) 24%/160 (4e-6) 26%/118 (2e-5) 29%/127 (2e-8) 25%/174 (9e-5)	Np_640175 AAL59670 AAM08054 AAG04692 NP_758683 NP_830922
SMR0027 TrhW	29556-31067	503	HCM1.99 (TrhW) R0007 (TrhW)	80.5%/502 (1.9e-147) 80.5%/502 (1.9e-147)	TrhW (pCAR1) TrhW (R391) TrhW (SXT) ORF216 (Rts1) TrhW (pSLT) TrhW (F. R100) RspH4268 Reu5572 (Ralstonia metallidurans) TrhW (pNL1) 20640 (Vibrio vulnificus) 20644 (Vibrio vulnificus)	25%/364 (9e-30) 27%/295 (1e-25) 28%/295 (4e-25) 29%/240 (1e-20) 23%/180 (2e-9) 25%/193 (8e-9) 26%/180 (2e-8) 21%/189 (0.001) 21%/184 (9e-8) 22%/198 (2e-6) 20%/176 (0.047)	NP_758684 AAM08011 AAL59671 BAB93778 AAL23484 AAA24689 ZP_00008319 ZP_00026548 NP_049159 NP_762593 NP_762597
SMR0028 TrhU	31206-32267	353	HCM1.100 (TrhU) R0006 (TrhU)	97%/334 (5.6e-145) 97%/334 (5.6e-145)	TrhU (SXT) TrhU (R391) ORF217 (Rts1) TrhU (pNL1) RspH4269 TrhU (pSLT) TrhU (pCAR1) TrhU (F. R100) Reu5571 20641 (Vibrio vulnificus)	36%/347 (1e-57) 36%/345 (2e-56) 31%/359 (5e-44) 33%/340 (1e-40) 30%/326 (1e-39) 31%/346 (2e-38) 32%/356 (3e-38) 32%/320 (1e-35) 27%/351 (7e-26) 30%/301 (8e-40)	AAL59673 AAM08014 BAB93779 AAD03953 ZP_00008320 AAL23495 NP_758685 AAA98088 ZP_00026547 NP_762594
SMR0029 TrhN	32286-35474	1062	HCM1.101 (TrhN) R0005 (TrhN)	87.6%/1062 (0) 87.6%/1062 (0)	ORF24 (Rts1) S005 (S. typhimurium) TrhN (SXT)	24%/856 (4e-47) 25%/988 (5e-42) 24%/497 (8-25);	BAB93806 AAK02035 AAL59755

SMR0030	c35503-36375	290	HCM1.106c R0004	75.9%/290 (1.2e-89) 75.9%/290 (1.2e-89)	TraN (R391)	29%/232 (9e-17) 24%/501 (3e-24); 28%/345 (6e-18) 30%/88 (5e-5) 27%/112 (0.008); 23%/134 (0.15) 25%/112 (0.032) 27%/111 (0.043); 24%/193 (0.63) 32%/67 (5e-4)	AAM07995 AAD03951 AAL23498 AAB61941 ZP_00008322 NP_762598
SMR0031 Trh	36555-38339	594	HCM1.107 (Trh) R0003 (Trh)	82.4%/592 (9.3e-199) 82.4%/592 (9.3e-199)	Reut_p_5684 DNA Helicase II (V. cholera) DNA Helicase II (S. marcescens) UvrD (S. typhimurium LT2) DNA Helicase II (S. typhi)	26%/586 (2e-38) 26%/641 (4e-35) 25%/638 (2e-32) 25%/643 (1e-29) 25%/643 (1e-29)	ZP_00026658 AAF93366 AAC46278 AAL22795 CAD07941
SMR0034	38734-39606	290	HCM1.108 R0002	60.1%/291 (1.2e-60) 60.1%/291 (1.2e-60)			
SMR0035	39864-40041	125	HCM1.109 R0001	87.2%/125 (3e-41) 87.2%/125 (3e-41)			
SMR0036	40102-41079	325	HCM1.110 R0210	56.9%/327 (5.1e-71) 56.9%/327 (5.1e-71)			
SMR0037	41137-42195	352	HCM1.111 R0209	75.7%/350 (8.2e-99) 75.7%/350 (8.2e-99)	s067 (SXT) ORF70 (R391) ORF101 (pCAR1) Ddes0370 ORF227 (Rts1) RspH2275 Reut163 NorQ (Paracoccus denitrificans) NorQ (Rhodobacter sphaeroides)	30%/276 (8e-24) 29%/275 (1e-23) 29%/238 (6e-17) 31%/132 (2e-10) 27%/224 (3e-8) 28%/179 (7e-8) 25%/159 (3e-6) 27%/153 (1e-6) 28%/175 (1e-5)	AAL59708 AAM08012 NP758644 ZP_00128764 BAB93789 ZP_00006359 ZP_00021263 AAA68972 AAC45373
SMR0038	42327-42500	57	HCM1.112 R0208	52.7%/55 (1.2e-7) 52.7%/55 (1.2e-7)			
SMR0039	42564-43691	375	HCM1.114 R0207	79.1%/378 (1.4e-106) 79.1%/378 (1.4e-106)	s068 (SXT) ORF72 (R391) Reut_p_5247 ORF228 (Rts1) Ddes0369 ORF102 (pCAR1) ORF74 (R391) ORF103 (pCAR1) sO70 (SXT) CobT (V. pestis pMT1) pHCM2.0017 (pHCM2) ORF233 (Rts1) NorD (R. sphaeroides) NorD (Paracoccus denitrificans) Saro_p_2284 (N. aromaticivorans) vWF type A domain (B. anthracis)	18%/234 (5e-4) 18%/234 (0.001) 15%/361 (0.004) 21%/251 (0.043) 21%/209 (0.71) 19%/233 (0.28) 22%/631 20%/537 (9e-11) 22%/330 21%/301 18%/706 21%/233 20%/194 18%/193 17%/597 14%/574	AAL59707 AAM08016 ZP_00026235 BAB93790 ZP_00128763 NP_758645 AAM08006 NP_758646 AAL59705 AAC13217 CAD09884 BAB93795 AAC45374 AAA68973 ZP_00095256 BA_1206
SMR0041	45848-46069	73	HCM1.116a R0205	65.8%/73 (5e-18) 65.8%/73 (5e-18)			

SMR0042	46183-47415	410	HCM1.117 R0204	78.5%/410 (9.6e-140) 78.5%/410 (9.6e-140)	ORF78 (R391) s072 (SXT) ORF146 (pCAR1) Reut_p_5650 Magn_p_1471 (M. magnetotacticum) ID498 (B. japonicum) Sma0911 (pSymA, S. meliloti) Vir. assoc. protein E (B. melilotis) DNA primase STM0901 Saro_p_3633 P4 DNA primase (STY4832) ORF229 (pNL1)	30%/368 (5e-27) 30%/372 (1e-25) 29%/370 (4e-19) 24%/314 (7e-9) 25%/258 (2e-6) 32%/187 (2e-7) 29%/177 (2e-6) 28%/224 (3e-5) 23%/299 24%/180 17%/287 19%/178	AAM08013 AAL59703 NP_756689 ZP_00026624 ZP_00048890 AAG60914 AAK65155 AAL52878 AAL19837 ZP_00096593 CAD06954 AAD03876
SMR0043	47685-48206	173	HCM1.118 R0203	69%/171 (2.9e-50) 69%/171 (2.9e-50)			
SMR0044	48285-49163	292	HCM1.119 R0202	51.45/292 (3.2e-59) 51.45/292 (3.2e-59)			
SMR0045	49233-50168	311	HCM1.120 R0201	52.4%/311 (9.5e-66) 52.4%/311 (9.5e-66)			
SMR0046	50237-51157	306	HCM1.121 R0200	55.3%/300 (1.8e-59) 55.3%/300 (1.8e-59)			
			HCM1.120 R0201	23%/95 (0.73) 23%/95 (0.73)			
SMR0047	51224-52162	312	HCM1.122 R0199	47.4%/310 (5.1e-52) 47.4%/310 (5.1e-52)			
			HCM1.123 R0198	26%/308 (8e-23) 27%/181 (e-8)			
			R0194	28%/125 (3e-7)			
SMR0048	52328-53290	320	R0198 R0194	66.6%/183 (8.6e-47) 53%/137 (3e-31)			
			R0199	26%/311 (3e-25)			
			HCM1.123	61%/318 (1e-102)			
			HCM1.122	27%/311 (2e-26)			
SMR0049	53524-53829	101	HCM1.124 R0193	50%/80 (8.3e-13) 50%/80 (8.3e-13)			
SMR0050	53874-54464	196	R0192 HCM1.125	62%/156 (6e-50) 62.8%/196 (1.5e-47)			
SMR0051	54472-54729	85	HCM1.126 R0191	64.7%/85 (6.3e-19) 64.7%/85 (6.3e-19)			
SMR0052	54803-55339	178	HCM1.127 R0190	57.9%/178 (2.1e-46) 57.9%/178 (2.1e-46)			
SMR0053	55356-55802	148	HCM1.128 R0189	57%/135 (e-34) 58%/135 (3e-35)			
SMR0055 Tus	55893-56855	320	HCM1.131 R0186	60.0%/316 (1.9e-71) 60.0%/316 (1.9e-71)	Tus (ORF15, Rts1) Tus (E. coli O157:H7) 1ECR Tus (S. flexneri) Tus (S. typhimurium LT2) STY1652 (S. typhi CT18) Tus (Y. pestis)	27%/154 (2e-4) 18%/149 18%/149 18%/190 17%/190 17%/174	BAB93578 NP_288045 AAN43216 AAL20390 CAD01897 AAF68950
SMR0056	56865-57770	301	HCM1.132 R0185	78.5%/302 (2.3e-101) 78.5%/302 (2.3e-101)			
SMR0057	57784-58050	88	HCM1.133	73.9%/88 (5.2e-27)			

SMR0058	c58072-59232	386	R0184 HCM1.134c R0183	73.9%/88 (5.2e-27) 64.8%/386 (1.7e-101) 64.8%/386 (1.7e-101)	CRE recombinase	27%/368 (4e-19)	P06956
SMR0059				Doubtful CDS			
SMR0060	59417-59629	70	R0182 (Hha) HCM1.135	91%/61 (2e-23) 90%/70 (7e-27)	Hha (E. coli) Haemolysin expression modulation protein STY0516 YdIA (R721) Hha (S. flexneri) Haem. modul. (E. coli O157:H7) HhaYmoA (Y. pestis) Hmo (S. flexneri, pWR501) Haem. modul. (L7004, pO157) STY1661 (S. typhi CT18)	50%/66 (9e-11) 50%/72 (8e-10) 52%/67 (9e-10) 50%/66 (9e-10) 50%/66 (9e-10) 58%/66 (1e-8) 42%/69 (5e-8) 42%/69 (2e-7) 44%/72 (5e-6)	S15270 CAD04958 BAB12630 AAN42060 NP_286201 NP_668376 AAK18587 AAC70072 CAD01906
SMR0061	59990-61072	360	HCM1.141 R0175	73.9%/360 (1.7e-110) 73.9%/360 (1.7e-110)	putative protein kinase (y3587, Y. pestis) Hso1308 (Haemophilus somnus) Hr0976 (Thermosynechococcus elongatus) Npun_p_3785 (Nostoc punctiforme) Tery_p_3358 (Trichodesmium erythraeum)	86%/499 (0) 59%/496 (0) 30%/416 (1e-109) 23%/372 (7e-84) 20%/375 (5e-62)	NP_670884 ZP_00123302 NP_681766 ZP_00108337 ZP_0074073
SMR0063	c62763-64403	546			y3586 (Y. pestis) Hsom1307 (H. somnus) Tery_p_1177 (T. erythraeum) alr2823 (Nostoc sp.) ECs2880 (E. coli O157:H7) SF2136 (S. flexneri) il0386 (T. elongatus)	47%/559 (1e-139) 36%/357 (1e-43) 26%/267 (0.057) 28%/213 (0.44) 23%/216 24%/216 29%/88 (1.5)	NP_670883 ZP_00123301 ZP_00072001 NP_486863 NP_310907 NP_707964 NP_681176
SMR0064	c64400-65440	346			y3585 (Y. pestis) Hsom1306 (H. somnus) y3581 (Y. pestis) Hsom1304 (H. somnus) Tery (R478) y3584 (Y. pestis) Hsom1305 (H. somnus) lir0974 (T. elongatus) Ecs2881 (E. coli O157:H7) Tery_p_3358 (T. erythraeum) alr2822 (Nostoc sp.) Npun_p_3783 (N. punctiforme) YegL (S. flexneri)	79%/286 (1e-140) 43%/351 (8e-73) 40%/196 (1e-35) 41%/178 (2e-32) 39%/181 (4e-29) 36%/185 (6e-25) 30%/199 (1e-23) 32%/200 (2e-19) 28%/157 (9e-11) 22%/313 (3e-10) 26%/199 (2e-9) 26%/199 (6e-9) 25%/144 (5e-6)	NP_670882 ZP_00123300 NP_670878 ZP_00123298 AAL44737 NP_670881 ZP_00123299 NP_681764 NP_310908 ZP_00074073 NP_486862 ZP_00108335 NP_707965
SMR0065	c65525-66163	212			y3584 (Y. pestis) Hsom1305 (H. somnus) Hsom1304 (H. somnus) y3581 (Y. pestis) Tery (R478) y3585 (Y. pestis) Hsom1306 (H. somnus) lir0974 (T. elongatus)	82%/212 (4e-96) 51%/211 (2e-59) 50%/216 (5e-50) 49%/190 (2e-45) 46%/202 (3e-37) 39%/194 (2e-31) 36%/197 (1e-23) 30%/223 (2e-18)	NP_670881 ZP_00123299 ZP_00123298 NP_670878 AAL44737 NP_670882 ZP_00123300 NP_681764

SMR0066	c6616-66804	213				TerY_p_3358 (T. erythraecum) all2822 (Nostoc sp.) TerY_p_1176 Npun_p_3783 (N. punctiforme) Ecs2881 (E. coli O157:H7) Chlor_p_2225 (Chloroflexus aurantiacus) YegL (S. flexneri) YvcC (Lactococcus lactis)	30%/198 (7e-15) 29%/199 (3e-11) 29%/213 (8e-11) 29%/199 (2e-10) 35%/121 (7e-10) 31%/98 (1e-4)	ZP_00074073 NP_486862 ZP_00072000 ZP_00109335 NP_310908 ZP_00019225
SMR0067	c66827-67420	197				y3583 (Y. pestis) many TerD and TerE homologues y3581 (Y. pestis) - also many from TerY2/3 including: Chlo_p_2225 (C. aurantiacus) TerW (Ecs1343, E. coli O157:H7) y0549 (Y. pestis)	32%/121 (2e-4) 26%/156 (7e-4) 86%/213 (1e-104)	NP_707965 NP_268200 NP_670879
SMR0068	c67928-68395	155				ECs1344 (E. coli O157:H7) ECs1345 (E. coli O157:H7) y0550 (Y. pestis) Pyr4495 (Pseudomonas syringae) Rub_p_1529 (Rhodospirillum rubrum) DR2219 (Deinococcus radiodurans) SCC8A.21C (Streptomyces coelicolor) RspH_p_3499 (R. sphaeroides)	85%/197 (2e-81) 30%/168 (1e-5) 99%/155 (9e-6) 51%/153 (2e-28) 98%/402 (0)	NP_670878 ZP_00019225 NP_309370 NP_667886 NP_309371
SMR0069	c68413-69621	402				ECs1344 (E. coli O157:H7) ECs1345 (E. coli O157:H7) y0550 (Y. pestis) Pyr4495 (Pseudomonas syringae) Rub_p_1529 (Rhodospirillum rubrum) DR2219 (Deinococcus radiodurans) SCC8A.21C (Streptomyces coelicolor) RspH_p_3499 (R. sphaeroides)	98%/318 (1e-179) 65%/310 (1e-115) 52%/298 (3e-72) 41%/313 (4e-54) 43%/300 (1e-53) 33%/353 (1e-31)	NP_309372 NP_667887 ZP_00128167 ZP_00014514 NP_295941 NP_626610
SMR0070	c69632-70588	318				ECs1344 (E. coli O157:H7) ECs1345 (E. coli O157:H7) y0550 (Y. pestis) Pyr4495 (Pseudomonas syringae) Rub_p_1529 (Rhodospirillum rubrum) DR2219 (Deinococcus radiodurans) SCC8A.21C (Streptomyces coelicolor) RspH_p_3499 (R. sphaeroides)	20%/305 98%/359 (0) 70%/353 (1e-147) 98%/224 (1e-136) 51%/352 (2e-97) 44%/356 (2e-75) 44%/356 (7e-63) 98%/106 (2e-54) 36%/346 (9e-44) 35%/335 (2e-33)	ZP_0007562 NP_287110 NP_667888 NP_309373 ZP_00128166 NP_659965 ZP_00014513 NP_309374 NP_626609 NP_295940
SMR0071	c70588-71667	359				Z1606 (E. coli O157:H7) y0551 (Y. pestis) Ecs1346 (E. coli O157:H7) Pyr4494 (P. syringae) yp063 (Rhizobium etli, p42d) Rub_p_1528 (R. rubrum) ECs1347 (E. coli O157:H7) SCC8A.20C (S. coelicolor) DR2218 (D. radiodurans)	28%/257 (1e-143) 66%/254 (1e-101) 41%/252 (5e-39) 35%/247 (6e-34) 37%/255 (1e-26) 33%/240 (2e-25) 21%/247	NP_309375 NP_667889 NP_659966 ZP_00128165 NP_295937 ZP_00014512 NP_626608
SMR0072	c71669-72442	257				ECs1348 (E. coli O157:H7) y0552 (Y. pestis) yp064 (Rhizobium etli, p42d) Pyr4493 (P. syringae) DR2215 (D. radiodurans) Rub_p_1527 (R. rubrum) SCC8A.19C (S. coelicolor)	98%/380 (0) 68%/372 (1e-145) 53%/361 (1e-103) 44%/371 (3e-78) 67%/198 (2e-76) 39%/368 (2e-53) 42%/216 (4e-34) 31%/349 (3e-16) 28%/177	NP_39376 NP_667890 ZP_00128164 NP_659964 AAD47283 NP_295936 ZP_00014511 NP_626609 ZP_0006173
SMR0073	c72435-73577	380				ECs1349 (E. coli O157:H7) y0553 (Y. pestis) Pyr4492 (P. syringae) yp062 (Rhizobium etli, p42d) ORF3 (Proteus mirabilis) DR2214 (D. radiodurans) Rub_p_1526 (R. rubrum) SCC8A.20c (S. coelicolor) RspH_p_2082 (R. sphaeroides)	98%/327 (0)	NP_309377
SMR0074	c73567-74576	329				ECs1350 (E. coli O157:H7)		

SMR0075 TerZ	74967-75548	193				y0554 (Y. pestis) Psr4490 (P. syringae) Rrub_p_1531 (R. rubrum) DR2213 (D. radiodurans) SCA7_21c (S. coelicolor) Npun_p_5173 (N. punctiforme) Reut_p_4570 (R. metallidurans) Magn_p_2486 (M. magnetotactum) SMB21227 carbamoyl phosphate synthase (S. meliloti, pSymB) TerZ ECs1351 (E. coli O157:H7) TerZ (Y. pestis) TerZ (P. mirabilis) Hsom1311 (H. somnus) Psr4497 (P. syringae) Rrub_p_1534 (R. rubrum) TerZ DR2224 (D. radiodurans) SCC54_25c (S. coelicolor) Npun_p_5790 (N. punctiforme) TerD BA_0974 (B. anthracis) YoeD (B. subtilis) Tery_p_2097 (T. erythraecum) Rrub_p_1533 (R. rubrum) TerD (Y. pestis) Hsom1310 (H. somnus) cAMP binding protein (Dictyostellium discoideum)	54%/320 (1e-98) 40%/285 (1e-54) 29%/287 (7e-23) 28%/292 (3e-14) 28%/288 (5e-14) 20%/252 (0.022) 26%/171 (0.055) 22%/181 (1.2) 21%/238	NP_667891 ZP_00128162 ZP_00014516 NP_295935 NP_630746 ZP_00110708 ZP_00025569 ZP_00049855 NP_437271
SMR0076 TerA	75548-76705	385				TerA (E. coli O157:H7) TerA (Ecs1352, E. coli O157:H7) TerA (Y. pestis) TerA (pMJ606, Alcaligenes sp.) TerA (P. mirabilis) Psr4498 (P. syringae) Rrub_p_1535 (R. rubrum) TerA DR2223 (D. radiodurans) TerA (pMER610, IncHI2) TerF (pTES3) SCC8A_23c (S. coelicolor) Npun_p_5789 (N. punctiforme)	99%/385 (0) 99%/341 (0) 68%/394 (1e-152) 75%/343 (1e-148) 65%/389 (1e-142) 49%/394 (6e-95) 38%/415 (3e-66) 37%/403 (7e-52) 74%/128 (2e-51) 44%/211 (5e-33) 36%/219 (3e-27) 36%/193 (4e-24)	NP_286707 NP_309379 NP_667893 AA98289 AAD47285 ZP_00128170 ZP_00014520 NP_29545 2009362A CAB43000 NP_626612 ZP_00111319
SMR0077 TerB	76728-77183	151				TerB (Ecs1353, E. coli O157:H7) TerB (pTES3) TerB (P. mirabilis) TerB (Y. pestis) TerB (pMJ606, Alcaligenes sp.) Avin_p_141 (Azotobacter vinelandii) Rrub_p_1536 (R. rubrum) Psr4499 (P. syringae) TerB DR2220 (D. radiodurans) SCH63_14 (S. coelicolor)	100%/151 99%/151 (5e-71) 81%/151 (8e-58) 71%/151 (1e-52) 92%/118 (2e-51) 43%/147 (5e-31) 43%/151 (1e-29) 42%/147 (2e-26) 325/151 (6e-15) 26%/151 (3e-10)	NP_309380 CAB42996 AAD47286 NP_667894 AA98290 ZP_00088494 ZP_00014521 ZP_00128171 NP_295942 NP_627957
SMR0078 TerC	77206-78246	346				TerC (Ecs1354, E. coli O157:H7) TerC (pMJ606, Alcaligenes sp.) TerC (P. mirabilis)	100%/346 90%/346 (1e-158) 88%/338 (1e-152)	NP_309381 AA98291 AAD47287

SMR0095	91753-92469	238	HCM1.213c HCM1.162c Transposase for IS26	100%/234 (e-126) 65%/233 (2e-77)	Cj0569 (Campylobacter jejuni) Cj0568 (C. jejuni) TnpIS26 (S. typhimurium pSEM) TnpIS15 (R391) ORF165 (Rts1) TnpA (linN R46) Reu0471 (R. metallidurans) Integrase (pseudogene) – 100% to C-term of numerous Int1 integrases (including from In2–R100/pNR) Recombinase/resolvase (pseudogene) Class III aminotransferase (pseudogene) (PDB avail.) Glutamine binding/transport (PDB) Glutamine permeases fragment of Glu ATP-bind component Alcohol dehydrogenase class III (PDB) STM1627 XAC0734 AdhC (E. coli O157:H7) ORF10 (Rts1)	29%/224 (2e-15) 33%/75 (0.74) 99%/238 (e-128) 99%/238 (e-128) 100%/234 (e-126) 100%/234 (1e-126) ?? (1e-36)	NP_281752 NP_281751 CAB71034 CAD27661 NP_640126 NP_511230 ZP_00021558	
SMR0096	92524-92841	105	-	-	-	-	-	
SMR0096a	92810-93049	80	-	-	-	-	-	
SMR0096b	93194-93340	49	-	-	-	-	-	
SMR0097	93392-94108	238	= SMR0095 100%	-	-	-	-	
SMR0098	94161-95089	312	-	-	-	-	-	
SMR0099	95096-95647	183	-	-	-	-	-	
SMR0100	95780-96532	250	-	-	-	-	-	
SMR0101	96585-97241	218	-	-	-	-	-	
SMR0102	97244-97330	29	-	-	-	-	-	
SMR0103	c97628-98344	238	= SMR0095/97	-	-	-	-	
SMR0104 AdhC	c98486-99604	372	-	-	-	-	94%/371 (0) 88%/369 (0) 81%/369 (1e-171) 80%/369 (1e-169) 87%/91 (1e-32) 57%/91 (2e-19) 57%/91 (2e-18) 53%/91 (5e-15) 49%/91 (4e-12) 40%/91 (3e-10) 43%/91 (9e-10) 35%/91 (8e-8)	NP_460586 NP_641287 NP_286099 NP_639971 NP_460587 NP_639970 NP_286100 NP_641086 NP_644358 NP_457418 ZP_0006630 ZP_00093770
SMR0105	c98650-99925	91	-	-	-	-	-	
SMR0106	100130-100459	109	-	-	-	-	-	
SMR0107	100620-101114	164	HCM1.207	99%/125 (2e-67)	ybA (R100) Smb21092 Bcep5068 XCC0457 XAC0474	99%/125 (2e-67) 31%/108 (8e-10) 30%/104 (4e-8) 31%/101 (1e-7) 36%/79 (1e-7)	NP_052902 NP_437211.1 ZP_00032220 NP_635851.1 NP_640829.1	
SMR0108 Cat	101315-101974	219	HCM1.206	99%/219 (1e-120)	cat (R100) and MANY other cat	99%/219 (1e-120)	NP_0529031	
SMR0109	c102196-102912	238	= SMR95/97/103	-	-	-	-	
SMR0110	c103018-103833	271	HCM1.223 StrA	27%/308	Identical to numerous aminoglycoside phosphotransferases	-	-	
SMR0112	103972-104688	238	=SMR95/97/103/109	-	-	-	-	
SMR0113	104743-105249	168	-	-	-	-	-	
SMR0114	105304-105567	88	HCM1.162c	100%/88 (2.7e-34)	TnpA (pseudogene via IS26 insert.) TnpA fragment (pseudogene) TnIA transposase fragment from IS21 (pseudogene)	100%/15	-	
SMR0115	c105627-105671	15	-	-	-	-	-	

SMR0115a	c105710-105775	21	HCM1.227c	100%/21	pseudogene	100%/70 (4e-25)	AA034401
SMR0116	c105808-106020	70	HCM1.161	100%/70 (4e-25)	UrtY (Mercury resist. bacteria) Reut5590	68%/70 (3e-20) 71%/69 (1e-26)	ZP_00026556 NP_052889.1
SMR0117	c106086-106322	78	HCM1.160 HCM1.229c	98%/78 (7e-32) 86%/78 (4e-26)	TnpM (Tn21 modulator, R100) Urt-1 (MerE, Alcaligenes sp.) Mer operon ORF1 (pDU1358) Reut5579	100%/78 (3e-32) 91%/78 (3e-30) 83%/77 (5e-27) 86%/78 (4e-26)	CAA70192.1 D29010 ZP_00026555 NP_052887.1 NP_361076.1
SMR0118	c106319-106705	128	HCM1.159 HCM1.230c HCM1.230c (MerR)	100%/121 (3e-49) 82%/121 (5e-32) 29%/105 (.005)	Urt1 (R100) MerE (pSB102) MerD (Alcaligenes sp.) MerD (pSB102) MerD (pDU1358) Reut5578	75%/78 (3e-23) 99%/121 (2e-48) 88%/126 (1e-47) 88%/121 (2e-43) 89%/128 (3e-43) 82%/121 (5e-32)	CAA70191.1 NP_361075.1 C29010 ZP_00026554 NP_052886.1 ZP_0005926
SMR0119	c106702-108387	561	HCM1.158 HCM1.231c	100%/561 (0) 84%/565 (0)	Raph1730 RspH3193 MerA (Klebsiella pneumoniae; 12604550) MerA (pKLH256 Enterobacter; 9159519) Reut5577 MerA (Tn501) MerA (pSB102) MerA (Alcaligenes sp.) MerA (R100) MerA (Tn21) Reut4216 MerA (pKLH201); 9159519 MerA (R391) Mer red fragment (pDU1358); 2666393 Serratia marcescens Mer. reductase	33%/108 (.002) 100%/561 (0) 99%/561 (0) 93%/561 (0) 93%/561 (0) 89%/561 (0) 88%/558 (0) 84%/565 (0) 84%/565 (0) 79%/559 (0) 100%/366 (0) 56%/364 (1e-163) 90%/343 (1e-162) 90%/342 (1e-161)	AA044226 CAA70231.1 ZP_00026553 NP_085424 NP_361073 CAA70190.1 NP_052885.1 AAC33905.1 ZP_00025223 CAA71183 AA080805 D33858 P08662.1
SMR0120	c108426-108851	141	HCM1.157 HCM1.232c	100%/141 (4e-63) 72%/140 (7e-39)	MerC (Klebsiella pneumoniae) MerC (pKLH256) MerC (pSB102) MerC (R100) MerC (Alcaligenes sp.); 9159519 MerC (R391)	100%/141 (4e-63) 100%/141 (4e-63) 75%/141 (9e-40) 74%/140 (5e-40) 68%/141 (4e-39) 59%/132 (6e-34)	AA044225 CAA70230.1 NP_361072 NP_052884 CAA70189.1 AA080859.1
SMR0121	c108879-109154	91	HCM1.153 HCM1.233c	100%/91 (3e-26) 82%/91 (7e-21)	MerP (Klebsiella pneumoniae) MerP (pKLH256) MerP (pDU1358) MerP (R100; PDB:1AFJ) Reut5576 MerP (RP1 Tn5053; 8594337) MerP (Alcaligenes sp.) MerP (pSB102) Reut4215 MerP (R391) Reut3236	100%/91 (3e-26) 100%/91 (3e-26) 83%/91 (4e-22) 83%/91 (5e-21) 85%/91 (8e-21) 84%/90 (8e-20) 85%/91 (1e-19) 82%/91 (7e-19) 67%/95 (1e-14) 54%/90 (1e-13) 50%/60 (5e-7)	AA044224 CAA70229.1 AA098223 NP_052883 ZP_00026552 S70144 CAA70197.1 NP_361070 ZP_00025222 AA080804.1 ZP_00024264
SMR0122	c109170-109535	121	HCM1.152 HCM1.234c	100%/121 (2e-53) 93%/115 (2e-47)	MerT (pKLH210) MerT (Klebsiella pneumoniae) MerT (pKLH256) MerT (Alcaligenes sp.) MerT (pSB102) Reut5575	100%/120 (7e-55) 100%/120 (7e-55) 100%/120 (3e-54) 99%/115 (5e-49) 95%/115 (4e-48) 89%/119 (1e-47)	CAA70239.1 AA044223 CAA70228 CAA70196 NP_361069 ZP_00026551

SMR0123	MerR	109607-110062	151	HCM1.151 HCM1.225	99%/151 (1e-79) 90%/142 (5e-65)	MerT (R100) MerT (pDU1358) Reut4214 Reut3237 MerT (R391) MerR (Alcaligenes sp.) MerR (pKLH201) MerR (pKLH256) MerR (Klebsiella pneumoniae) Tn501 repressor, 11292750 Reut0554 Reut5574 MerR (pSB102) MerR (Tn5053) MerR (R100) MerR (pDU1358) Reut4213 Reut3238 MerR (R391) Sma0281 (pSymA) RspH3193 MerR (Exiguobacterium sp. pKLH3; 9534232)	93%/115 (2e-47) 96%/115 (2e-45) 81%/115 (3e-38) 55%/110 (2e-23) 52%/100 (8e-20) 100%/151 (4e-80) 100%/151 (4e-80) 100%/151 (4e-80) 99%/151 (8e-80) 92%/142 (2e-68) 92%/142 (2e-67) 92%/142 (2e-67) 89%/142 (5e-67) 90%/142 (5e-65) 92%/133 (7e-64) 74%/127 (4e-48) 52%/122 (8e-28) 45%/124 (2e-25) 46%/128 (3e-21) 39%/128 (3e-20) 38%/125 (1e-17)	NP_052882.1 AAA98222.1 ZP_00025221 ZP_00024265 AAM08065.1 CAA70195 CAA70240.1 CAA70237.1 AAM44222.1 NP_085422.1 ZP_00021641 ZP_00026550 NP_361068.1 S32798 NP_052881.1 AAA98221.1 ZP_00025200 ZP_00024266 AAM08061.1 NP_435397 ZP_00007262 CAA67818
SMR0124		110128-110736	202	HCM1.177 HCM1.175 R0166 R0168	53%/194 (2e-53) 36%/99 (4e-4) 59%/118 (1e-35) 36%/99 (4e-4)	ORF5 (E. coli O6:K15:H31; 12379716) Reut5304	29%/143 (2e-7) 33%/77 (0.056)	CA033715.1 ZP_00026287
SMR0125		110798-111001	67			Sih (Shigella)	79%/134 (1e-37)	AAN38340
SMR0126	H-NS	c111343-111747	134	HCM1.178ac R0164 (H-NS)	81%/134 (1e-37) 81%/134 (2e-37)	H-NS (Erwinia) StoA (E. coli) H-NS (E. coli O157:H7, 1HNR, 1LR1_A) StoA (S. Typhi) H-NS (Yersinia) H-NS (S. Typhi) Reut0246 KorB (IncN R46 regulator) CR1 (Citrobacter LEE regul., 11553577)	60%/135 (2e-33) 62%/134 (8e-33) 62%/134 (8e-33) 59%/134 (5e-30) 60%/136 (3e-29) 63%/138 (5e-26) 34%/103 (8e-5) 43%/53 (0.003) 20%/123	CAC44357 CAA49146 NP_288219 NP_457198 CAC24849 NP_455749.1 ZP_00021345 NP_511185.1 AAL06349
SMR0127		c111925-112284	119			ORF21 (Citrobacter freundii pCTX-M3, broad host-range conj. plasmid)	31%/135 (3e-10)	NP_774991.1
SMR0128		c112521-112976	151	HCM1.176 R0167	24%/131 24%/131	ORF7 (pCTX-M3) ORF163 (Rts1)	40%/210 (1e-37) 36%/214 (2e-29)	NP_774973.1 NP_640124.1
SMR0129		c113036-113701	221	HCM1.180c R0161	57%/218 (9e-68) 57%/218 (9e-68)	Tn7 TnsA (1400257, 2166235; 1F1Z_A, 1F1Z_B) Chie1624 (Clostridium thermocellum) Npun0417 Tn7 TnsB Reut3107 Npun4141 Chie1623	39%/192 (4e-27) (last 80 aa don't match) 28%/667 (2e-56) 25%/669 (2e-45) 25%/695 (2e-42) 27%/459 (3e-29) 33%/313 (7e-37) 34%/373 (1e-51)	NP_065320.1 ZP_00061216 ZP_00106048 NP_065319.1 ZP_08024138 ZP_00109790 ZP_00061215
SMR0130		c113759-114139	126					
SMR0132		114891-115733	280					
SMR0133		115765-117843	692					
SMR0134		117843-119291	482					

SMR0135	119323-120888	521				Npun4242 Tn7 TnsC	31%/377 (3e-51) 30%/383 (1e-33)	ZP_00109791 NP_065318.1
			-			Npun4245 Chie1622 Tn7 TnsD	28%/177 (9e-8) 28%/110 (2e-4) 23%/156 (0.095); 21%/206 (0.21)	ZP_00109794 ZP_00061214 NP_065317.1
SMR0136	120900-121826	308						
SMR0138	122179-122478	99				HipB (R. solanacearum megaplasamid) Bcep7822 TM1330 (Thermoboga maritima)	44%/81 (4e-8) 36%/68 (2e-4) 26%/80 (7e-4)	NP_522813.1 ZP_00034923 NP_229132.1
SMR0139	123042-124868	608				ORF024 (pMD136) ORF04 (pMD136) ORFT (ICES1) S024 (S. typhimurium) Au5023 (pAT) SMC (R. sphaeroides) msi092 (M. loti symbiosis island, 12003951)	36%/556 (4e-86) 40%/1213 (2e-34) 21%/566 (2e-8) 29%/173 (1e-7) 25%/398 (3e-7) 47%/44 (7e-4) 26%/209(0.002); 30%/85 (0.023)	NP_037556.1 NP_052600.1 CAC67530.1 AAK02041 NP_535400 CAD66603 CAD31497.1
SMR0140	c125037-125387	116				mif5326 (M. loti) Bcep2139 SMa1024 (pSymA) SMc00921 Magn4014 Chio2357	45%/114 (1e-13) 41%/108 (8e-11) 45%/91 (8e-10) 47%/80 (2e-9) 38%/88 (1e-8) 31%/105 (6e-5)	NP_106012.1 ZP_00029342 NP_435800.1 NP_384890.1 ZP_00051328 ZP_00019356
SMR0141	c125535-125966	143				SlIE (S. typhimurium, pMG101) SlIE (R476b) 11739772 SlIE (pMIP233) SlIE (pMIP235) SlIE (pWR23) PcoE (pRJ1004, cop bind., 8594334) STY0609 (copper binding)	100%/143 (2e-54) 100%/127 (2e-48) 100%/127 (2e-48) 100%/127 (2e-48) 92%/127 (1e-44) 47%/142 (1e-16) 35%/82 (0.014)	AA011743.1 AAC68931.1 AAC68941.1 AAC68944.1 AAC68950.1 CAA58532.1 NP_455145
SMR0142	c126211-127692	493				SlIS (pMG101) 9930866 YbcZ (E. coli) CopS (E. coli K-12) SlIS (R476b) SlIS (pMIP233) SlIS (pWR23) SlIS (pMIP235) Reut5289/ CopS PcoS (pRJ1004)	93%/497 (0) 56%/480 (e-144) 56%/480 (e-144) 100%/198; 100%/133 99%/198; 99%/133 99%/198; 98%/130 98%/198; 99%/133 32%/475 (2e-65) 29%/465 (9e-52)	AA011744 NP_415102 BAA35204.1 AAL68929.30 AAL68939.40 AAL68951.52 AAL68945.46 ZP_00026274 CAA58530
SMR0143	c127685-128365	226				SlIR (pMG101) CopR (E. coli K12) YcIA (E. coli) Reut5288/CopR PcoR (pRJ1004) Reut3251/CzrR CopR (S. typhi CT18)	82%/228 (1e-108) 87%/225 (e-105) 87%/225 (e-105) 67%/225 (4e-76) 58%/222 (5e-65) 55%/222 (2e-64) 54%/222 (2e-59)	AA011745 BAA35205 NP_415103 ZP_00026273 CAA58529 CAA67086 NP_455593
SMR0144	128555-129940	461				SlIC (pMG101) YicB (E. coli) OprK (E. coli K12) CusC (E. coli, 11004187)	97%/461 (0) 71%/450 (e-173) 71%/450 (e-173) 69%/341 (e-123)	AA011746 NP_415104 BAA35206 AAE70174
SMR0145	129968-130321	117				ORF96 (pMG101)	96%/90 (e-38)	AA011747.1

SMR0146 SiB	130435-131727	430	-	-	-	YdC (E. coli) CusX (E. coli)	47%/117 (e-18) 46%/117 (e-18)	NP 415105 NP 752580
SMR0147 SIA	131738-134884	1048	-	-	-	SiIB (pMG101) YdD (E. coli) CusB (E. coli) CzcB (P. putida) Reut5289	96%/430 (0) 67%/409 (e-158) 60%/409 (e-157) 39%/383 (2e-72) 37%/375 (e-62)	AAD11748.1 NP 415106 NP 752581 NP 747487 ZP 00026255
SMR0148	134971-135411	146	-	-	-	SiIA (pMG101) YbdE (E. coli) CusA (E. coli) CzcA (P. putida) Reut5288	98%/1048 (0) 87%/1044 (0) 87%/1044 (0) 66%/1047 (0) 66%/1035 (0)	AAD11749.1 NP 415107 NP 752592 NP 747488 ZP 00026254
SMR0149 SiP	135538-137985	815	-	-	-	ORF105 (pMG101) SMA1008 (pSymA) SMB20881 (pSymB) PP5377 (P. putida) Reut5281/CopG	96%/105 (3-50) 43%/141 (e-24) 41%/130 (5e-23) 40%/138 (2e-19) 37%/119 (e-14)	AAD11752.1 NP 435790 NP 437715 NP 747478 ZP 00026267
SMR0150	138026-138223	65	-	-	-	SiIP (pMG101) SMA1087 (pSymA) Reut5280 ActP (Rhizobium leguminosarum) SMB21578	95%/816 (0) 66%/733 (0) 53%/808 (0) 54%/808 (0) 49%/679 (1e-164)	AAD11750 NP 435835 ZP 00026266 AAD26860 NP 437588
SMR0151	138257-138994	245	-	-	-	PP5390 (P. putida) SMA1088 (pSymA) ORF191 (pMG101) YebA (S. typhi) YebA (E. coli)	57%/38 (2e-4) 62%/53 (0.13) 94%/172 (2e-81) 40%/210 (6e-41) 40%/210 (7e-41)	NP 747491 NP 435836 AAD11751 NP 456457 H64947
SMR0152 CopE2	139283-139732	149	-	-	-	SiIE (pMG101) pccE (pRJ1004)	34%/118 (2e-9) 35%/142 (6e-6)	AAD11743 CAA58532
SMR0153 CopA	139967-141784	605	-	-	-	pccA (pRJ1004) McoA (K. pneumoniae) Pyr4025 Reut5287	99%/605 (0) 99%/605 (0) 73%/610 (0) 54%/613 (0)	CAA58525 AAN52531 ZP 127705 ZP 00026272 CAC07979
SMR154 CopB	141784-142680	298	-	-	-	CopA (R. metallidurans) pccB (pRJ1004) Pyr4024 Reut5285	54%/613 (0) 100%/296 (e-168) 60%/264 (e-91) 44%/224 (6e-47)	CAA58526 ZP 127704 ZP 00026271 CAC07890
SMR0155 CopC	142720-143100	126	-	-	-	CopB (R. metallidurans) pccC (pRJ1004) Reut5285	44%/225 (8e-27) 100%/126 (2e-56) 64%/114 (e-30)	CAA58527 ZP 00026270 CAC07981
SMR0156 CopD	143105-144034	309	-	-	-	CopC (R. metallidurans) Pyr4023 pccD (pRJ1004) Pyr4022	63%/112 (8e-27) 100%/309 (e-139) 38%/305 (5e-46)	ZP 127703 CAA58528 ZP 127702
SMR0157 CopR	144089-144769	226	-	-	-	Reut5284 CopD (R. metallidurans) SMA1198 (pSymA) pccR (pRJ1004) Pyr4021 Yica (E. coli) Reut5286 czrR (R. metallidurans)	41%/306 (6e-44) 39%/306 (6e-40) 28%/255 (1.5) 100%/226 (e-118) 60%/226 (3e-67) 60%/222 (7e-67) 63%/225 (3e-66) 57%/220 (2e-63)	ZP 00026269 CAC07982 NP 435900 CAA58529 ZP 127701 NP 415103 ZP 00026273 CAC67086

SMR0158 Cops	144766-146166	466	-	-	-	pcoS (pRJ1004) Reut5289 Cops (R. metallidurans) YbcZ (E. coli) Pyr4020 Sils (pMG101) czcS (R. metallidurans)	99%/466 (0) 33%/460 (4e-64) 32%/460 (2e-63) 29%/474 (3e-55) 31%/451 (e-51) 28%/470 (4e-49) 28%/451 (e-42)	CAA58530 ZP_00026274 CAC07977 NP_415102 ZP_127700 AAD11744 CAA67087
SMR0159 Cope1	144766-146166	144	-	-	-	pcoE (pRJ1004) Sile (pMG101)	92%/144 (2e-33) 45%/142 (e-15)	CAA58532 AAD11743
SMR0160	147196-148014	272	HCM1.181 R0160 HCM1.182 R0159	60%/151 (e-47) 60%/151 (e-47) 67%/115 (4e-42) 67%/115 (4e-42)	ORF26 (pCTX-M3, C. freundii) ORF20 (pWWO, P. putida) yda (pColib-P9) Raph4197 ORF62 (Rts1) HCM2.0104c	22%/281 (4e-12) 23%/259 (2e-11) 25%/207 (4e-8) 24%/224 (5e-8) 24%/208 (0.14) 18%/227	NP_774997 NP_542813 NP_052499 ZP_00008253 NP_640023 NP_568576	
SMR0161	148011-149216	401	lipA (R0158) HCM1.183	40%/406 (5e-57) 40%/406 (5e-57)	<20% identity to numerous proteins (bacterial and eukaryotic)			
SMR0162	149280-149483	67	HCM1.184c R0157	59%/67 (2e-13) 59%/67 (2e-12)				
SMR0163	149496-150815	439	HCM1.185c R0156	81%/431 (0) 81%/431 (0)	ORF6 (pWWO) ORF544 (pNL1) SMA0901 (pSymA) mir9256 (pMLA) yp034 (p42d, R. eti) Reut5628	36%/434 (2e-19) 25%/348 (3e-15) 23%/382 (3e-15) 25%/334 (e-13) 22%/381 (4e-12) 23%/361 (8e-7)	NP_542801 NP_049116 NP_435741 NP_085787 NP_659881 ZP_00026603	
SMR0164	150838-151005	55	HCM1.186c R0155	61%/55 (3e-11) 61%/55 (4e-11)				
SMR0165 Dcm	151066-152493	475	HCM1.187c R0154	72%/460 (0) 71%/460 (0)	EcoRII cytosine methylase Dcm (E. coli)	67%/430 (e-175) 64%/438 (e-162)	CA28725 NP_416470	
SMR0166	152708-153223	171	R0153 (parB) HCM1.188	74%/170 (e-64) 72%/170 (6e-64)	ParB (RP4) Nuc (pUCD5409, S. flexneri)	37%/166 (8e-25) 36%/160 (1e-21)	AAA26415 AAA75246	
SMR0167	153226-154122	298	HCM1.189 R0152	51%/297 (2e-87) 51%/297 (2e-87)	ORF151 (Rts1)	22%/254 (8e-5)	NP_640112	
SMR0168	154148-154378	76	-	-	-	-	-	-
SMR0170	154874-155107	77	HCM1.191	56%/60 (3e-16)	-	-	-	-
SMR0171	155445-155732	95	HCM1.192	33%/66 (e-6)	-	-	-	-
SMR0172	155769-155999	76	-	-	-	-	-	-
SMR0173	156140-156415	91	-	-	-	-	-	-
SMR0174 Sipa	156907-158385	492	-	-	ORF280 (Rts1) ORF3 (Y. enterocolitica plasmid frag.) ORF89 (Rts1) ORF51 (R391) ORFA (Tn1404) 10543801 Reut2691 Raph4233 SMB20070 (pSymB) SMF20070 (Y. enterocolitica plasmid frag.) ORF88 (Rts1) ORFB (Tn1404) ORF52 (R391) Reut2690 Raph4234	43%/89 (2e-11) 99%/492 (0) 76%/492 (0) 74%/495 (0) 74%/494 (0) 74%/494 (0) 65%/491 (e-166) 65%/483 (e-158) 97%/275 (e-140) 43%/278 (3e-52) 43%/282 (8e-49) 36%/283 (e-44) 36%/284 (2e-37) 31%/188 (e-16)	NP_640240 CAA73749 NP_640050 AAM08007 AAD47994 ZP_00023729 ZP_00008287 NP_436610 CAA73748 NP_640049 AAD47991 AAM08025 ZP_00023728 ZP_00008288	
SMR0175	158404-159231	275	-	-	-			

SMR0176 AraC	c159291-159716	141	-	-	-	AraC (R773) AraC (E. coli) AraC (R46)	90%/140 (3e-66) 88%/140 (2e-65) 87%/140 (4e-64)	AAA21096 NP_417980 NP_511240
SMR0177 AraB	c159729-161018	429	-	-	-	AraB (R46) AraB (E. coli) AraB (R773) Psr4015	86%/429 (e-167) 86%/429 (e-168) 85%/429 (e-164) 73%/413 (e-129)	NP_511239 NP_417959 AAA21095 ZP_00127695
SMR0178 AraR	c161064-161364	106	-	-	-	AraR (E. coli) AraR (R733) AraR (R46) Psr4014 Rsp4238	63%/105 (e-33) 58%/105 (e-30) 56%/102 (4e-29) 43%/64 (2e-11) 42%/71 (2e-9)	P_47958 BVECAR NP_511236 ZP_00127694 ZP_00008292
SMR0179 AraH	161471-162175	234	-	-	-	AraH (P. putida) Psr4017	74%/204 (5e-83) 69%/231 (2e-87)	ZP_00127687 NP_744859
SMR0180	c162208-163611	467	HCM1.201 R0148	73%/460 (0) 96%/465 (0)	-	Y4bF (pNGR234) Trs1202 ORF167 (Rts1)	47%/425 (e-101) 28%/328 (6e-28) 81%/49 (e-14)	AAB91621 O54513 NP_640127
SMR0181	163803-164120	105	-	-	-	-	-	-
SMR0182	164143-164448	101	-	-	-	Weakly similar to serine proteases	-	-
SMR0183	164493-165164	223	-	-	-	-	-	-
SMR0184	165165-165422	85	-	-	-	-	-	-
SMR0185	165622-166029	135	HCM1.197c R0143	55%/127 (4e-38) 55%/127 (4e-38)	-	-	-	-
SMR0186	c166080-166607	175	-	-	-	-	-	-
SMR0187	c166774-167154	126	-	-	-	-	-	-
SMR0188	c167227-170562	1111	HCM1.195 R0140	46%/1112 (0) 45%/942 (0)	-	ORF164 (Rts1) ORF173 (Rts1)	47%/449 (e-114) 41%/668 (2e-94)	NP_640125 NP_640133
SMR0189	c170785-171138	117	HCM1.250c R0139	28%/103 (e-4) 29%/114 (2e-8)	-	-	-	-
SMR0190	c171292-171900	202	HCM1.251c R0138	50%/202 (3e-50) 52%/130 (8e-30)	-	-	-	-
SMR0191 RelA	c172114-173601	495	-	-	-	RelA (R471a, S. marcescens) MalRa (pNL1) SMB21167 (pSymb) Many more intron-associ. maturases	100%/495 (0) 425%/211 (e-36) 32%/377 (e-34)	AAC82519 NP_049088 CAC49233
SMR0192 MucA	174003-174437	144	HCM1.252 R0137	81%/144 (4e-63) 81%/144 (2e-62)	-	MucA (pCTX-M3) MucA (R446b) MucA (R471a) ORF7 (Rts1) MucA (R394) 11106794 MucA (R46) MucA (pKM101) RumA (R391) ORF79 (Rts1)	56%/119 (2e-31) 56%/119 (e-31) 56%/119 (e-31) 55%/124 (5e-28) 55%/124 (6e-28) 52%/121 (5e-26) 52%/121 (5e-26) 48%/118 (e-20) 41%/122 (6e-20)	NP_774966 AAC82517 AAC82521 NP_639968 AAF14052 NP_511216 AAB36886 AAM08055 NP_640040
SMR0193 MucB	174427-175680	417	HCM1.253 R0136	83%/417 (0) 83%/402 (0)	-	MucB (pCTX-M3) MucB (R446b) MucB (R471a) MucC (E. coli) MucB (R46) MucB (pKM101) RumB (R391)	44%/411 (e-105) 45%/411 (e-105) 44%/411 (e-104) 48%/417 (e-102) 46%/408 (e-101) 46%/408 (e-101) 43%/416 (7e-93)	NP_774967 AAC82518 AAC82522 NP_415702 NP_511217 AAD17384 AAM08023

SMR0194	c175726-176535	269	HCM1.254c R0135		ORF80 (Rts1) MucB (R394) ORF8 (Rts1)	38%/415 (8e-78) 45%/325 (e-76) 45%/325 (2e-76)	NP_640041 AAF14053 NP_639969
SMR0195	176642-177253	203	HCM1.255 R0134		bcH (S. typhimurium) STY0033 (S. typhi)	28%/275 (5e-24) 28%/275 (6e-24)	NP_459033 NP_454638
SMR0196	c177313-177735	140	HCM1.256c R0133				
SMR0197	c177769-178161	130	HCM1.257c R0132				
SMR0198 DsbC	c178186-178935	249	HCM1.258c R0131		DsbC (Erwinia sp.)	35%/246 (6e-36)	CAA54108
SMR0199	179083-179622	179	HCM1.259 R0130 (bipH)		bipH (pB171) trnN (pB4) trnN (X. fastidiosa) trnN (R751) trnN (RP4) trnN (pCTX-M3) Reut5666 lagB (S. typhi, cell invasion protein)	35%/135 (8e-15) 35%/116 (5e-13) 31%/158 (8e-13) 33%/119 (1e-12) 32%/119 (4e-12) 34%/120 (2e-9) 33%/114 (2e-9) 30%/117 (2e-5)	NP_053074 NP_590155 NP_779548 NP_044252 AAA26440 NP_775063 ZP_00026640 NP_457271
SMR0200	179900-180262	120	HCM1.261 R0129				
SMR0201	180386-181447	353			YP0258 (Y. pestis) yhhZ (E. coli) YraA (E. coli K-12) y3542 (Y. pestis)	50%/216 (2e-53) 46%/234 (6e-51) 26%/136 (4e-7) 23%/105 (0.009)	NP_404857 NP_417899 NP_417900 NP_670839
SMR0202	181457-181963	168					
SMR0203 ThG	c182086-186036	1316	HCM1.262 R0128 (ThG)		S011 (S. typhimurium) TraG (pED208, S. typhimurium) TrhG (pCART, Pseudomonas resinovorans) ORF242 (Rts1) TraG (SXT) TraG (R391) 20649 (Vibrio vulnificus)	22%/836 (7e-28) 23%/564 (2e-4) 31%/134 (0.37) 20%/106 (1e-25) 19%/1050 (6e-16) 18%/1045 (4e-15) 20.8%/1090	AAK02037 AAM90723 NP_758692 NP_640202 AAL59677 ~ AAM07986 NP_762602
SMR0204 ThH	c186045-187460	471	HCM1.263c R0127 (ThH)		TraH (R391) TraH (SXT) TraH (R100) TrhH (pCART1) 12547188 TraH (pED208) S012 (S. typhimurium) ORF241 (Rts1) TraH (F) TraH (pSLT) Raph_p_4274 (R. sphaeroides) TraH (Neisseria gonorrhoeae) TraH (pNL1) 20648 (Vibrio vulnificus)	28%/297 (2e-25) 27%/297 (3e-24) 26%/425 (7e-22) 26%/418 (3e-21) 26%/362 (6e-17) 26%/341 (4e-20) 30%/258 (3e-19) 26%/354 (1e-17) 26%/355 (2e-16) 22%/363 (4e-12) 22%/452 (2e-11) 21%/455 (1e-10) 25%/383 (2e-17)	AAM08008 AAL59676 AAD28727 NP_758691 AAM90722 AAK02038 NP_640201 AAA98080 NP_490584 ZP_00008325 AAK77138 AAD03948 NP_762601
SMR0205 ThF	c187450-188496	348	HCM1.264c R0126 (ThF)		TraF (R391) TraF (SXT) ORF240 (Rts1)	29%/278 (4e-33) 29%/281 (8e-33) 26%/321 (7e-25)	AAM08018 AAL59678 NP_640200

SMR0206	c189088-189600	170	HCM1.266c R0124 (TmY)	85.9%/170 (3e-57) 85.9%/170 (3e-57)	TraF (pCAR1) TraF (pED208) TraF (pNL1) RspH_p_4273 (R. sphaeroides) TraF (F) TraF (pSLT) 20646 (Vibrio vulnificus) 20647 (Vibrio vulnificus)	31%/292 (2e-24) 25%/259 (2e-12) 24%/201 (4e-12) 19%/233 (3e-6) 20%/219 (4e-6) 21%/228 (2e-4) 20%/224 (8e-5) 24%/104	NP_758690 AAM90720 AAD03949 ZP_00008324 AAB61943 NP_490581 NP_762599 NP_762600
SMR0207	c189602-190402	266	HCM1.268c R0123 (TmR)	77.8%/266 (8.3e-85) 77.8%/266 (8.3e-85)	-	-	-
SMR0208	190950-191111	53	-	-	DR0518 (D. radiodurans) (86 aa)	57%/26 (5.5)	NP_294241
SMR0209	191162-191647	161	HCM1.269 R0122 (TmH)	76.4%/161 (8.5e-49) 76.4%/161 (8.5e-49)	-	-	-
SMR0210	191644-192381	245	HCM1.270 R0121	68.2%/245 (5.9e-57) 68.2%/245 (5.9e-57)	-	-	-
SMR0211 TraI	192385-195537	1050	HCM1.271 R0120 (TraI)	63%/1060 (0.0) 63%/1060 (0.0)	TraI (SXT) TraI (R391) TraI (pCAR1) ORF201 (Rts1) ORFSG44 (P. aeruginosa) Hsom1101 (H. somnus) XAC2196 (Xanthomonas axonopodis pv. citri) Pili_p_5158 (Pseudomonas fluorescens) Reut_p_2852 (R. metallidurans) Boep_p_3583 (Burkholderia fungorum) PHG365 (pHG1, Ralstonia eutropha) Reut5678 (R. metallidurans)	31%/261 (4e-18) 31%/244 (5e-18) 31%/239 (1e-18) 30%/203 (7e-14) 40%/66 (8e-7) 33%/127 (1e-7) 35%/92 (5e-7) 39%/66 (9e-7) 36%/105 (6e-6) 34%/105 (4e-4)	AAL59675 AAM08003 NP_758664 NP_640161 AAN62266 ZP_00123099 NP_642513 ZP_00087872 ZP_00023889 ZP_00030759 AAP86114 ZP_00026652 AAL59680 AAM08004 ZP_00026653 AAP86115 NP_640162 NP_758665 NP_458648 AAN62290 ZP_00123135 ZP_00030778 NP_542873 ZP_00025244 ZP_00087890 NP_642576 ZP_00127721 CAA44852 CAA75326
SMR0212 TraG	195537-197621	694	HCM1.272 R0119 (TraG)	83%/694 (0.0) 83%/694 (0.0)	TraD (SXT) TraD (R391) Reut_p_5679 (R. metallidurans) TraG (pHG1) ORF202 (Rts1) TraG (pCAR1) STY4562 ORFSG68 (P. aeruginosa) Hsom1138 (H. somnus) Boep_p_3602 (B. fungorum) ORF106 (pWWO, P. putida) Reut_p_4237 (R. metallidurans) Pili_p_5177 (P. fluorescens) XAC2259 (X. axonopodis pv. citri) Peyr4044 (P. syringae) TrwB (R388) lcmO (Leigionella pneumophila)	16%/238 16%/233 31%/542 (9e-60) 31%/542 (1e-59) 26%/664 (3e-48) 26%/655 (2e-46) 26%/551 (2e-30) 27%/540 (e-42) 24%/628 (7e-30) 24%/652 (1e-28) 25%/553 (3e-28) 24%/632 (4e-28) 23%/585 (2e-27) 24%/626 (1e-27) 23%/655 (9e-27) 25%/449 (3e-26) 23%/635 (1e-21) 44%/38 (1.3) 22%/244 (1.5)	-
SMR0213	197618-198730	370	HCM1.273 R0118	65%/370 (e-144) 65%/370 (e-144)	-	-	-
SMR0214	198717-199379	220	HCM1.275	81.4%/220 (2.8e-70)	s043 (SXT)	31%/96 (4.9)	AAL59721

TraJ			R0117 (TraJ)	81.4%/220 (2.8e-70)	ORF36 (R391) ORF124 (pCAR1) ORF204 (Rts1) ORF105 (pWWO) STY4563 (S. typhi) SG67 (P. aeruginosa) Hsom1136 (H. somnus) Pilu5176 (P. fluorescens) PHG369 (pHG1) Reu4238 (R. metallidurans) Beep3601 (B. fungorum) Reut5682 (R. metallidurans) Psyn4045 (P. syringae) XAC2260 (X. axonopodis pv. citri)	30%/96 (7.0) 28%/46 21%/184 18%/158 16%/178 17%/160 18%/154 16%/154 14%/158 14%/159 14%/159 13%/169 18%/127 20%/89	AAM08039 NP_758667 NP_640164 NP_542872 NP_458649 AAN62289 ZP_00123133 ZP_00087889 AAP86118 ZP_00025245 ZP_00030777 ZP_00026656 ZP_00127722 NP_642577
SMR0215	199380-200571	393	HCM1.276 R0116	72%/393 (e-162) 72%/393 (e-162)	sppA (P. syringae) * and numerous other proteases	44%/125 (e-19) and 29%/131 (0.003)	NP_793611
SMR0216	200573-201304	243	HCM1.277 R0115	62%/242 (2e-88) 62%/242 (2e-88)	BXA0003 (pXO1-01)	32%/115 (2e-11)	NP_652769
SMR0217	c201591-201944	117	HCM1.278c R0114	73%/116 (7e-41) 73%/116 (7e-41)	ORF143 (pCAR1)	29%/144 (e-8)	NP_758686
SMR0218	c202010-202294	94	HCM1.279c R0113	55%/60 (e-8) 55%/60 (e-8)	-	-	-
SMR0219 Tn10R	202920-204128	402	HCM1.194 R0085	99%/402 (0) 99%/401 (0)	-	-	-
SMR0220 TelD	c204138-204554	138	R0084 TelD	100%/138 (3e-61)	>97% identity to numerous eukaryotic and bacterial transposases	-	-
SMR0221 TelC	204642-205235	197	R0083 TelC	100%/197 (e-101)	TelD (S. flexneri) TelD (E. coli) Rob (E. coli) 10802742, 1D5Y YkgA (E. coli) SoxS (S. typhi) MarA (E. coli) 9724717 TelC (S. flexneri) * numerous others ~30 identity	100%/138 (3e-61) 98%/138 (e-60) 60%/104 (3e-31) 40%/122 (4e-20) 50%/102 (5e-20) 45%/102 (2e-17) 100%/197 (e-101)	AAD50249 TETD_ECOLI NP_418813 NP_752353 NP_418486 NP_418048 AAD50248
SMR0222 TelA	c205348-206553	401	HCM1.241c R0082 TelA	99%/401 (0) 99%/401 (0)	TelA (S. flexneri) * highly conserved antiporter	100%/401 (0)	AAD50247
SMR0223 TelR	206635-207258	207	HCM1.243 R0081 TelR	100%/207 (e-112) 100%/207 (e-112)	TelR (S. flexneri) * highly conserved repressor	100%/207 (e-112)	AAD50246
SMR0224	c207236-207922	228	HCM1.244c R0080	99%/228 (e-122) 100%/228 (e-122)	JemC (S. flexneri) * ~30% identity to ArsR-family transcriptional repressors	100%/228 (e-122)	AAD50245
SMR0225	c07930-208316	128	HCM1.245c R0079	100%/128 (e-62) 100%/128 (e-63)	YdJB (R100) BMEI0153 (Brucella melitensis)	100%/128 (5e-63) 50%/127 (6e-28)	NP_052926 NP_539071
SMR0226	c208309-208629	106	HCM1.246c R0078	100%/106 (e-49) 100%/106 (e-49)	YdJA (R100) JemB (S. flexneri) BMEI0152	100%/106 (e-49) 100%/106 (e-49) 53%/100 (5e-23)	NP_052927 AAD50244 NP_539070
SMR0227 Gls	209073-210278	401	R0077 GlS HCM1.247 (psuedogene)	99%/77 (0)	YdHA (R100) JemA (S. flexneri) * highly conserved transporter	99%/77 (0) 99%/77 (0)	NP_052928 AAD50243
SMR0228 Tn10L	c210644-211852	402	HCM1.249c R0076	100%/402 (0) 99%/402	>97% identity to numerous eukaryotic and bacterial transposases	-	-
SMR0230	c212539-212907	122	HCM1.196 R0142	45%/132 (e-20) 45%/132 (e-20)	-	-	-

SMR0231	c213133-213669	178	HCM1.280c R0100	63%/177 (3e-58) 63%/177 (3e-58)	-	-	-	-
SMR0232	c213737-214163	138	HCM1.281c R0109	54%/136 (2e-34) 54%/136 (2e-34)	-	-	-	-
SMR0233	c214219-214515	98	-	-	-	-	-	-
SMR0234	c214650-215351	233	R0107	55%/61 (3e-11)	-	-	-	-
SMR0235	c215666-215947	93	HCM1.284c R0105	61%/93 (6e-22) 61%/93 (6e-22)	-	-	-	-
SMR0236	c216013-217197	394	HCM1.286c R0104	65%/403 (e-131) 65%/403 (e-131)	ParB (Caulobacter vibrioides)	41%/60 (0.020)	-	AAB51268
SMR0237	c217614-217844	76	-	-	-	-	-	-
SMR0238	218301-219245	314	-	-	-	-	-	-
SMR0239	219344-219943	199	-	-	-	-	-	-
SMR0240	220003-220353	116	-	-	-	-	-	-
SMR0241	c220489-220812	107	-	-	-	-	-	-
SMR0242	220885-221205	105	-	-	-	-	-	-
SMR0243	221857-222969	370	-	-	-	-	-	-
SMR0243a	c223163-223321	52	HCM1.290c R0101 Sm	86%/52 (2e-16) 86%/52 (2e-17)	IS186 Tn (E. coli) IS231 Tn (B. thuringiensis) ORF39 (pX01) Hok (R100) FimA (F plasmid)	99%/370 (0) 23%/385 (6e-9) 22%/287 (2e-8) 69%/52 (9e-12) 69%/52 (9e-12)	-	NP_416895 CAD30116 NP_052735 NP_052939 AAA99216 NP_418015 NP_794751 NP_720882 NP_688077 AAF73806 NP_403776 NP_052891
SMR0245	c223420-224271	283	-	-	-	-	-	-
SMR0246	c224268-224789	173	-	-	-	-	-	-
SMR0247	c225122-225727	201	R0100 HCM1.01c	42%/198 (9e-39) 44%/175 (7e-34)	IS150 Tn (E. coli) ISPsy9 Tn OrfB ISSnu1 Tn IS861 Tn (S. agalactiae) IS3-Spm1 Tn IS1661 (Y. pestis) IS1353 (R100)	100%/283 (e-156) 58%/272 (2e-89) 51%/274 (2e-67) 50%/272 (3e-66) 47%/269 (5e-61) 48%/258 (8e-60) 39%/279 (2e-54)	-	NP_418014 NP_403777 NP_794752
SMR0248	c225944-226225	93	HCM1.02c R0099	76%/86 (2e-29) 76%/86 (7e-29)	-	-	-	-
SMR0249	c226601-226912	103	HCM1.03c R0098	60%/87 (5e-26) 60%/87 (5e-26)	ORF281 (Rts1)	30%/105 (0.002)	-	NP_640251
SMR0250	c227135-227335	66	HCM1.10c R0093	58%/55 (e-9) 58%/55 (e-9)	-	-	-	-
SMR0251	c227375-227599	74	HCM1.13c R0092	29%/41 (2.1) 29%/41 (2.1)	-	-	-	-
SMR0251a	c227654-227857	67	HCM1.15c	79%/74 (4e-26) 79%/74 (4e-26)	-	-	-	-
SMR0252	c228410-228601	163	HCM1.20c	50%/63 (e-9) 50%/63 (e-9)	-	-	-	-
				28%/67 (0.0064) 51%/162 (9e-45)	STY4595	41%/58 (0.005)	-	NP_4586789

SMR0253	c228806-229217	103	R0089	51%/162 (4e-44) 73%/100 (3e-28) 73%/100 (3e-28)	ORF23 (pCTX-M3) ORF61 (E. coli 536) * conserved in many E. coli strains	24%/90 48%/49 (e-8)	NP_774983 CAD66209
SMR0254	c229734-230054	106	-	-	-	-	-
SMR0255	c230233-230463	76	HCM1_23c R0086	57%/68 (9e-14) 57%/68 (9e-14)	-	-	-
SMR0256	c230635-231528	297	R0074	32%/95	KiaC (pCAR1)	23%/263 (0.22)	NP_758600
SMR0257	c231518-232618	366	-	-	TaiA (RK2 Te-r) KiaB (pCAR1) YaaN (B. subtilis) ORF182 (Rts1) * many tellurite resistance proteins	56%/359 (e-104) 57%/356 (e-101) 30%/340 (7e-37) 29%/368 (9e-33)	AAA98154 NP_758599 NP_387907 NP_640142
SMR0258	c232627-233412	261	-	-	KiaA (RK2 Te-r) KiaA (pCAR1) ORF183 (Rts1)	45%/256 (3e-61) 41%/249 (4e-50) 19%/202 (0.008)	AAA98153 NP_758598 NP_640143
SMR0259	Join c233643- 233798 to c233802-233930	94	HCM1_25c	67%/95 (2e-24)	-	-	-
SMR0261	c234007-234615	202	HCM1_26c R0074 (1-42)	62%/201 (9e-57) 69%/142 (5e-9)	PA2593 (P. aeruginosa) Avin0923	34%/112 (9e-8) 38%/47 (2e-4)	NP_251283 ZP_00089256
SMR0262	c234729-235262	177	HCM1_27c	79%/174 (5e-174)	SF1348 (S. flexneri)	45%/135 (7e23)	NP_707245
SMR0263	c235317-235511	64	-	-	-	-	-
SMR0264	c235508-235819	103	HCM1_28c	70%/98 (2e-34)	-	-	-
SMR0265	c235882-236121	79	HCM1_30c R0072 HCM1_13c R0092	69%/66 (e-14) 69%/66 (e-14) 51%/70 (5e-14) 51%/70 (5e-14)	-	-	-
SMR0266	c236371-238755	794	HCM1_32c R0070	76%/792 (0) 75%/791 (0)	abiG17 (Anopheles gambiae, contam?) air4917 (Nostoc sp.) SAV2951 (Streptomyces avermitilis)	40%/635 (e-137) 28%/319 (5e-17) 30%/291 (3e-15)	EAA01880 NP_488957 NP_824107
SMR0267	c238921-239370	149	HCM1_33c R0069	61%/144 (e-43) 61%/144 (3e-43)	-	-	-
SMR0268	c239421-240212	263	HCM1_34c R0068	59%/264 (4e-81) 59%/264 (4e-81)	-	-	-
SMR0269	c240442-240699	85	HCM1_35c R0067	45%/55 (4e-5) 46%/45 (0.026)	-	-	-
SMR0270	c240765-241091	108	HCM1_36c R0066	62%/106 (e-33) 62%/106 (e-33)	-	-	-
SMR0271	c241334-241654	106	-	-	-	-	-
SMR0272	c241949-243199	416	-	-	SpA1M (S. paratyphi) Pvull (P. vulgaris)	31%/314 (e-33) 30%/393 (4e-31)	AAG42425 CAA32026
SMR0273	c243370-243978	202	-	-	CPE0665 (C. perfringens) SCC24.26c (S. coelicolor) RSp1337 (R. solanacearum)	30%/82 (6e-6) 32%/98 (4e-4) 27%/98 (0.017)	NP_561581 NP_626698 NP_522896
SMR0274	c244134-244406	90	-	-	-	-	-
SMR0275	c244456-244800	115	-	-	-	-	-
SMR0276	c244976-245353	125	-	-	-	-	-
SMR0277	c245759-246940	393	HCM1_38c R0065	62%/392 (e-143) 61%/316 (e-114)	-	-	-

SMR0278	c246949-247245	98	HCM1.39c	89%/98 (4e-39)	VCA0391 SMC02461 H1gB (R1s1, ORF261)	43%/95 (e-11) 29%/98 (e-4) 30%/93 (0.01)	NP_232785 NP_385157 NP_640221
SMR0279	c247295-247765	156	HCM1.40c R0063	64%/155 (2e-47)		-	-
SMR0280	c248199-250589	796	HCM1.49c R0055 HCM1.56c R0045 R0047 HCM1.52 R0051 HCM1.50 R0054	64%/155 (2e-47) 38%/787 (e-139) 38%/787 (e-139) 29%/843 (8e-69) 26%/723 (e-35) 49%/108 (2e-23) 41%/130 (3e-20) 40%/125 (4e-18) 30%/160 (5e-13) 31%/138 (e-12)	ORF131 (pCAR1)	23%/805 (3e-33)	NP_758674
SMR0281	c250992-251243	83	-	-	-	-	-
SMR0282	c251451-251708	85	-	-	-	-	-
SMR0283	c251701-252132	143	-	-	ORF281 (R1s1)	38%/130 (2e-7)	NP_640241
SMR0284	c252236-252760	174	-	-	-	-	-
SMR0285	252776-252916	46	-	-	-	-	-
SMR0286	253083-253847	254	R0060 HCM1.43 HCM1.42 R0061	61%/255 (9e-85) 61%/255 (1e-84) 32%/264 (2e-29) 32%/264 (2e-29)	-	-	-
SMR0287	253865-254689	274	HCM1.57c R0043	29%/141 (9e-8) 29%/141 (e-7)	Reph4197 Unnamed (pO157) ydcA (R100) ORF139 (R1s1) HCM2.0104c	29%/186 (5e-12) 29%/131 (2e-10) 29%/131 (8e-10) 27%/153 (2e-8) 30%/190	NP_82537 NP_052654 NP_052920 NP_640100 NP_569576 NP_640247
SMR0288	254783-255046	87	HCM1.45 R0058	66%/87 (5e-29) 66%/87 (e-28)	ORF287 (R1s1)	31%/87 (7e-4)	-
SMR0289	255090-255533	147	-	-	-	-	-
SMR0290	255767-258187	806	HCM1.56c R0045 R0055 HCM1.49c R0054 HCM1.50 HCM1.52 R0051 R0047 HCM1.51 R0052 R0053 HCM1.53	42%/814 (e-176) 39%/710 (e-136) 31%/812 (6e-89) 31%/812 (9e-89) 70%/202 (3e-75) 71%/200 (e-74) 85%/131 (2e-56) 86%/131 (5e-56) 60%/112 (2e-30) 74%/81 (4e-28) 74%/81 (4e-28) 85%/57 (2e-21) 86%/45 (3e-15)	ORF131 (pCAR1)	29%/799 (2e-86)	NP_758674
SMR0292 Reph12	c260265-261353	362	R0036 RepH1A HCM1.64 RepA2 R0050 RepH1B HCM1.54 RepA	43%/285 (5e-59) 43%/285 (5e-59) 38%/276 (2e-46) 38%/276 (2e-46)	RepA (pHCM2) RepA (pMT1, Y. pestis) RepA (R386) RepA (pSLT) RepFIB (F plasmid) RepA (Vir plasmid, S. choleraesuis) RepA (pO157) RepA (P1 phage)	51%/333 (e-83) 51%/346 (e-82) 52%/283 (5e-71) 50%/308 (5e-71) 52%/283 (9e-71) 49%/316 (9e-71) 52%/274 (2e-68) 41%/269 (2e-53)	NP_569603 NP_395385 AAA71884 NP_490513 AAA71886 NP_073241 NP_052630 BVBPA

SMR0293	c262461-264917	818	HCM1.56c R0045 R0055 HCM1.49c R0047 R0051 HCM1.52 HCM1.50 R0054 R0052 HCM1.51 HCM1.53 R0053	81%/818 (0) 81%/705 (0) 35%/836 (3e-99) 35%/834 (6e-99) 83%/112 (4e-46) 56%/127 (3e-31) 52%/129 (9e-31) 40%/196 (3e-28) 39%/200 (9e-28) 45%/81 (e-10) 45%/81 (e-10) 54%/46 (7e-6) 38%/54 (0.001)	RepA (pSW1200, Pantoea stewartii) RepA (Rts1) RepA1 (pS268a, Serratia sp.) RepA2 (pS268a, Serratia sp.) ORF131 (pCAR1)	43%/257 (2e-52) 41%/271 (3e-52) 42%/140 (3e-24) 43%/128 (2e-22) 30%/826 (e-100)	AAB66323 NP_639962 AAL84745 AAL84746 NP_758674
SMR0294	265290-265916	209			ORF4 (pCTX-M3) Atu0367 1 and numerous other methionyl-tRNA formyl transferases (new domain at 98- 156?)	44%/206 (7e-47) 41%/53	NP_774970 NP_513073
SMR0295	c265974-266255	93					
SMR0296	c266242-266885	147					
SMR0297	c266746-266922	58	HCM1.56ac R0044	61%/57 61%/57			
SMR0298	c266919-268610	563	HCM1.57c R0043	70%/558 (0) 70%/558 (0)	HCM2.104c ORF62 (Rts1) ORF139 (Rts1) ORF26 (pCTX-M3)	30%/574 (2e-67) 28%/522 (e-49) 28%/485 (2e-45) 28%/235 (9e-12)	NP_569576 NP_640023 NP_640100 NP_774997
SMR0299	c268858-270021	387	HCM1.58c R0042 HCM1.286c R0104 HCM1.63c R0037	72%/384 (e-159) 72%/384 (e-159) 30%/236 (9e-16) 29%/231 (2e-15) 29%/200 (2e-11) 29%/200 (2e-11)	ORF112 (pCAR1) ORF273 (Rts1) ParB (Chlamydomonas reinhardtii)	28%/243 (2e-9) 24%/230 (2e-7) 24%/85 (0.019)	NP_758655 NP_640233 NP_828930
SMR0300 Dam	270370-271203	277	HCM1.59c R0041	70%/272 (e-111) 70%/257 (e-102)	STY1014 ORF53 (Rts1) ORF131 (Rts1) * >30% id to many DNA adenine methylases	39%/270 (2e-45) 35%/267 (9e-38) 34%/265 (7e-32)	NP_455493 NP_640014 NP_640092
SMR0301	c271212-272063	283	HCM1.60 R0040	63%/277 (2e-95) 63%/277 (2e-95)			
SMR0302	c272301-273017	238	HCM1.61c R0039	53%/231 (7e-63) 53%/231 (7e-63)			
SMR0303	c273014-273214	66	HCM1.62c R0038	53%/66 (3e-10) 51%/66 (5e-10)			
SMR0304	c273234-274286	350	HCM1.63c R0037 HCM1.58c R0042 HCM1.286c	58%/355 (5e-90) 58%/355 (5e-90) 25%/298 (9e-16) 25%/298 (9e-16) 27%/195 (5e-7)	ORF112(pCAR1) ParB (Treponema pallidum) ORF273 (Rts1)	20%/189 (6e-4) 26%/165 (0.002) 23%/143 (0.33)	NP_758655 NP_218712 NP_640233

Curriculum Vitae: Matthew W. Gilmour

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EDUCATION:

- ▲ Ph.D. - Department of Medical Microbiology and Immunology (University of Alberta); September 1999 - March 2004, Supervisor: Dr. Diane Taylor
 - ▲ Thesis defense examination passed February 26th, 2004
 - ▲ Project: Determination of the essential genes, protein function, and protein-protein interactions required for the conjugative transfer (i.e. Type IV secretion) of R27, a 180 kbp resistance plasmid from *Salmonella enterica* serovar Typhi
 - ▲ Project: Comparative genomics of IncHI plasmids and related conjugative genetic elements. The IncHI2 plasmid R478 isolated from *Serratia marcescens* is 275 kbp, confers multiple drug resistance, and shows sequence identity to numerous other self-transmissible elements. My goals are to annotate the R478 sequence data to reveal the function and the evolutionary history of backbone and accessory genes, and to compare the IncHI1 plasmid backbone features to those encoded in the IncHI2 plasmid in order to determine the minimal set of determinants for plasmid maintenance and conjugative transfer. These aims are being achieved by using Sanger Institute software Artemis and ACT for the annotation and comparative analysis of genomes.
 - ▲ Project: Commissioned to write with Dr. Diane Taylor a chapter in the new online version of EcoSal (see www.ecosal.org - formerly called '*Escherichia coli* and *Salmonella*: Cellular and Molecular Biology', edited by Neidhardt *et al.*); section '*Molecular Architecture of Bacterial Cells*'; subsection '*Cytology*'; tentative chapter title: '*Cytology of Bacterial Conjugation*' (editor: Dr. B.B. Finlay).
 - ▲ Project: Collaboration with the research groups of Samuel Kaplan (University of Texas) and Frank Larimer (Oak Ridge National Laboratory) to complete the annotation of the *Rhodobacter sphaeroides* 2.4.1 genomic sequence. My particular role is to annotate the coding sequences of the 5 large plasmids present in the sequenced strain.

- ▲ Experimental procedures routinely used include: bioinformatic analysis of DNA sequences, molecular DNA techniques, protein purification through affinity chromatography, fluorescence microscopy, bacterial two-hybrid analysis, cellular fractionation, functional analysis of purified proteins, and site-directed mutagenesis of whole genes and specific motifs
- ▲ B.Sc. with Specialization in Cell Biotechnology (with Distinction); June 1999, University of Alberta
 - ▲ This undergraduate program was a combination of both the Microbiology and Genetics programs offered by the Department of Biological Sciences, with a focus on obtaining formal training in laboratory techniques
 - ▲ Instructional laboratories include: Microbiology Laboratory Techniques (protein and DNA purification), Microbial Physiology Laboratory (metabolism of microorganisms), Introduction to Molecular Genetics Techniques (Molecular and Cellular biology), Bacterial Pathogenesis Laboratory (clinical analysis of bacterial isolates), Fermentation Biotechnology (large-scale fungal and bacterial cultures and product purification)
 - ▲ Thesis project: characterization of the membrane-associated protein TrhC required for bacterial conjugative transfer of plasmid R27 (Supervisor: Dr. Diane Taylor)
 - ▲ Received Dean's Honor Roll placement in third and final years of the program

PUBLICATIONS (Refereed):

- 1) **Gilmour MW** and Taylor DE. 2004. A subassembly of R27-encoded transfer proteins is dependent on TrhC nucleotide triphosphate-binding motifs for function but not formation. *Journal of Bacteriology*. 186(6):1606-13.
- 2) **Gilmour MW**, Gunton JE, Lawley TD, Taylor DE. 2003. Interaction between the IncHI1 plasmid R27 coupling protein and Type IV secretion system: TraG associates with the coiled-coil Mating pair formation protein TrhB. *Molecular Microbiology*. 49(1):105-16.
- 3) Lawley TD, **Gilmour MW**, Gunton JE, Tracz DM, Taylor DE. 2003. Functional and mutational analysis of conjugative transfer region 2 (Tra2) from the IncHI1 plasmid R27. *Journal of Bacteriology*. 185(2):581-91.
- 4) Lawley TD, **Gilmour MW**, Gunton JE, Standeven LJ, Taylor DE. 2002. Functional and mutational analysis of conjugative transfer region 1 (Tra1) from the IncHI1 plasmid R27. *Journal of Bacteriology*. 184(8):2173-80.
- 5) **Gilmour MW**, Lawley TD, Rooker MM, Newnham PJ, Taylor DE. 2001. Cellular location and temperature-dependent assembly of IncHI1 plasmid

R27-encoded TrhC-associated conjugative transfer protein complexes.
Molecular Microbiology. 42(3):705-15.

- 6) Sherburne CK, Lawley TD, **Gilmour MW**, Blattner FR, Burland V, Grotbeck E, Rose DJ, Taylor DE. 2000. The complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. *Nucleic Acids Research*. 15;28(10):2177-86.

MANUSCRIPTS (Submitted for publication):

- 1) **Gilmour MW**, Thomson NR, Sanders M, Parkhill J, Taylor DE. The complete nucleotide sequence of R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. Submitted to the *Journal of Molecular Biology* October 2003.

PUBLISHED ABSTRACTS

- 1) **Gilmour MW** and Taylor DE. Assembly requirements of TrhC-associated protein complexes which are involved in the conjugative transfer of R27. Poster presented at Plasmid Biology 2002. Pittsburgh, Pennsylvania, USA. June 22-28, 2002. Abstract published in *Plasmid*. 48(3):252.
- 2) Gunton JE, Lawley TD, **Gilmour MW**, Taylor DE. Characterization of the coupling protein of the IncHI1 plasmid R27. Poster presented at Plasmid Biology 2002. Pittsburgh, Pennsylvania, USA. June 22-28, 2002. Abstract published in *Plasmid*. 48(3): 256.
- 3) Tracz DM, Lawley TD, **Gilmour MW**, Gunton JE, and Taylor DE. Characterization of the Tra1 and Tra2 conjugative transfer regions of the IncHI1 plasmid R27: defining a type IV secretion system subfamily. Poster presented at Plasmid Biology 2002. Pittsburgh, Pennsylvania, USA. June 22-28, 2002. Abstract published in *Plasmid*. 48(3): 282.
- 4) **Gilmour MW**, Lawley TD, Gunton JE, Tracz DM, and Taylor DE. Characterization of the Tra1 and Tra2 conjugative transfer regions of the IncHI1 plasmid R27: defining a type IV secretion system subfamily. Poster presented at the UA-UC Conference on Infectious Diseases 2002. Banff, Canada. April 21-24, 2002. Abstract #1.
- 5) **Gilmour MW**, Lawley TD, Rooker M, Gunton JE, and Taylor DE. Fusion of green fluorescent protein to the conjugal transfer protein TrhC: localization and temperature-dependent expression. Poster presented at the EURESCO conference on the Biology of Type IV Secretion Processes: Euroconference

on the Medical and Ecological Implications. Lucca, Italy. September 7-12 2001. pg.74.

- 6) **Gilmour MW**, Lawley TD, Sherburne CK, Blattner FR, Burland V, Grotbeck E, Rose DJ, and Taylor DE. Highlights of the complete DNA sequence and analysis of R27, a large IncHI plasmid from *Salmonella typhi* that is temperature sensitive for transfer. Poster presented at the International Symposium on Molecular Biology of Bacterial Plasmids. Prague, Czech Republic. September 19-25, 2000. Abstract published in *Plasmid* 45(2):164-165.

INVITED PRESENTATIONS/VISITS:

- 1) University of British Columbia (Vancouver, Canada): March 16th, 2003; host: Dr. Brett B. Finlay
- 2) National Microbiology Laboratory (Winnipeg, Canada): June 4th, 2003; host: Dr. Lai-King Ng
- 3) The Sanger Institute (Wellcome Trust Genome Campus, Cambridge, UK): August 17-29, 2003; hosts: Dr. Julian Parkhill and Dr. Nick Thomson
- 4) University of Texas (Houston, USA); December 4th, 2003; host: Dr. Peter Christie

CONFERENCES/WORKSHOPS ATTENDED:

- 1) Canadian Bioinformatics Workshops: BIOINFORMATICS 2003, Vancouver, Canada. February 2003. Intensive two-week program with lectures/tutorials/laboratories focussing on databases, webpage design, sequence submission, sequence alignment, unix/linux platforms, BLAST, sequence analysis, phylogeny, PERL programming, RNA sequence analysis, and protein structural analysis. (Final grade: 91%, A+ standing)
- 2) Plasmid Biology 2002. Pittsburgh, USA. June 2002. Poster presented.
- 3) UA-UC Conference on Infectious Diseases 2002. Banff, Canada. Poster presented.
- 4) EURESCO conference on the Biology of Type IV Secretion Processes. Lucca, Italy. September 2001. Poster presented.
- 5) International Symposium on the Biology of Plasmids. Prague, Czech Republic. September 2000. Poster presented.

GRANTS & AWARDS:

- 1) 2003-2004. Honorary Izaak Killam Memorial Scholarship. This is the highest honour awarded to Graduate Students by the University of Alberta, and the award component covers tuition, a \$2600 funding increase, and a \$2000 research allowance
- 2) 2003. Andrew Stewart Memorial Graduate Student Prize. \$5000
- 3) 2002-2007. Canadian Institutes of Health Research operating grant (awarded to D.E. Taylor, principal investigator, and T.D. Lawley and M.W. Gilmour, co-applicants)
- 4) 2000-present. Alberta Heritage Foundation for Medical Research Studentship. \$20000/year + \$1500 research allowance/year
- 5) 2000. J Gordon Kaplan Graduate Student Award. \$800
- 6) 1995. Alexander Rutherford Scholarship. \$1500

INSTRUCTIONAL EXPERIENCE:

- ▲ Teaching Assistant for MMI 520 (Biology of Plasmids); 2000-present. Duties include lecturing students, grading presentations and grant proposals, and preparing and marking exams
- ▲ Teaching Assistant for MMI 351 (Bacterial Pathogenesis Laboratory); 2000-present. Duties were same as MMI 520, plus supervision of students
- ▲ Direct supervision and instruction of junior graduate students (James Gunton, Leah Standeven, and Dobryan Tracz) and undergraduate project students (Jamil Janmohamed, Eugene Lam, and Andrew Ting)

REFERENCES:

- 1) Dr. Diane Taylor (diane.taylor@ualberta.ca; 780-492-4777)
- 2) Dr. Laura Frost (laura.frost@ualberta.ca; 780-492-0672)
- 3) Dr. Glen Armstrong (glen.armstrong@ucalgary.ca; 403-220-6885)
- 4) Dr. Markus Stein (mstein@ualberta.ca; 780-492-5495)